FACULTY OF VETERINARY MEDICINE
accredited by EAEVE

WHEAT BRAN WITH REDUCED PARTICLE SIZE AS CARRIER FOR MICROBIAL NETWORKS THAT AFFECT SALMONELLA COLONIZATION

KAREN VERMEULEN

A dissertation submitted to Ghent University in partial fulfilment of the requirements for the degree of Doctor of Philosophy (PhD) in Veterinary Sciences

2017

PROMOTORS:
Prof. Dr. ir. Filip Van Immerseel,
Prof. Dr. Richard Ducatelle

Faculty of Veterinary Medicine
Department of Pathology, Bacteriology & Avian Diseases

GHENT UNIVERSITY

This research was funded by “Vlaams Agentschap Innoveren en Ondernemen” (Vlaio)
SBO BRANDING, grant no. 130028

Cover by Daan Tanghe
Printed by University Press, Zelzate, Belgium

“When you come out of the storm, you won’t be the same person who walked in.
That’s what this storm is all about.”

[Haruki Murakami, Kafka on the Shore]

LIST OF ABBREVIATIONS

LIST OF ABBREVIATIONS

Ara	Arabinose
AX	Arabinoxylan
AXOS	Arabino-xylo-oligosaccharides
BPW	Buffered peptone water
CAZy	Carbohydrate active enzymes
CFU	Colony forming units
CE	Carbohydrate esterase
CoAt	Butyryl-CoA: acetate CoA-transferase gene
CTAB	Cetyl trimethyl ammonium bromide
DDGS	Distillers dried grains with solubles
DF	Dietary Fibre
DMEM	Dulbecco modified eagle medium
DSPE	Destarched pericarp enriched
ED	Entner – Doudoroff pathway
EDA	2-keto 3-deoxy-D-gluconate 6-phosphate aldolase
EDD	6-phosphogluconate dehydratase
EMP	Embden – Meyerhof pathway
FCR	Feed conversion rate
FCS	Fetal calf serum
FerA	Ferulic acid
FOS	Fructo-oligosaccharides
Gal	Galactose
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GF	Germ free
GH	Glycoside hydrolase
GIT	Gastrointestinal tract
GLP-2	Glucagon-like peptide 2
GLP-2	Glucagon-like peptide 2 receptor
Glu	Glucose
GluA	Glucuronic acid
GOS	Galacto-oligosaccharides

HBSS	Hanks' balanced salt solution
IFNγ	Interferon γ
LB	Luria - Bertani broth
MCFA	Medium chain fatty acids
ME	Metabolizable energy
MOS	Mannan-oligosaccharides
NE	Necrotic enteritis
NFκB	Nuclear factor κB
NOD	Nucleotide oligomerization domain
NSP	Non starch polysaccharides
OS	Oligosaccharides
OTU	Operational taxonomic unit
PBS	Phosphate-buffered saline
PEP	Phosphoenolpyruvate
PFK	Phosphofructokinase
PL	Polysaccharide lyase
PP	Pentose phosphate pathway
PPARγ	Peroxisome proliferator-activated receptor γ
RS	Resistant starch
SCFA	Short chain fatty acids
SCV	*Salmonella* containing vacuole
SD	Standard deviation
SFB	Segmented filamentous bacteria
SPI-1	*Salmonella* pathogenicity island 1
T3SS	Type 3 secretion system
TLR	Toll-like receptor
WB	Wheat bran
WB280	Wheat bran with reduced average particle size of 280 µm
XLD	Xylose lysine deoxycholate
XOS	Xylo-oligosaccharides
XUS	Xylan utilization system
Xyl	Xylose

PART 1

GENERAL INTRODUCTION

1. DIETARY FIBRE

1.1 DEFINITION

In 1953 the term 'dietary fibre' (DF) was introduced for the first time by Dr. Eben Hipsley to describe indigestible constituents of the plant cell wall (Hipsley 1953). In the following decades, numerous definitions of DF have been proposed (DeVries et al. 1999, McCleary 2011). In 2008, the Codex Alimentarius Commission adopted a comprehensive definition (Codex Alimentarius Commission 2009) which describes DF as follows:

"*Carbohydrate polymers[1] with ten or more monomeric units[2], which are not hydrolyzed by the endogenous enzymes in the small intestine of humans and belong to the following categories:*

- *Edible carbohydrate polymers naturally occurring in the food as consumed.*
- *Carbohydrate polymers, which have been obtained from food raw material by physical, enzymatic or chemical means and which have been shown to have a physiological effect of benefit to health as demonstrated by generally accepted scientific evidence to competent authorities,*
- *Synthetic carbohydrate polymers, which have been shown to have a physiological effect of benefit to health as demonstrated by generally accepted scientific evidence to competent authorities."*

Footnote 1 states, "when derived from a plant origin, dietary fibre may include fractions of lignin and/or other compounds associated with polysaccharides in the plant cell walls. These compounds also may be measured by certain analytical method(s) for dietary fibre."
Footnote 2 states that, "Decision on whether to include carbohydrates of three to nine monomeric units should be left up to national authorities."

The European Commission published an almost identical definition for DF. However the definition was slightly different because of the inclusion of nondigestible oligosaccharides with three to nine monomeric units (EU 2008). This definition reflects the

current state of knowledge on DF and recognizes that all substances that behave like fibre can be classified as DF provided that they show physiological benefits (DeVries et al. 1999, Jones 2014).

1.2 CLASSIFICATION

Several classification systems for DF have been proposed, however, none is adequate since the borders cannot be strictly defined (Tungland 2002). Often, naturally occurring DFs are classified based on their chemical composition. This system subdivides DF in non-starch polysaccharides and resistant oligosaccharides (cellulose, hemicellulose, polyfructoses, galacto-oligosaccharides, gums, pectins and mucilages), resistant starches, lignin and plant substances which are associated with the non-starch polysaccharides and lignin network (e.g. waxes, phytate, cutins and saponins) (American Association of Cereal Chemists 2001). Using an alternative approach, DF can also be classified based on their physiochemical properties. Verspreet et al. (2016) proposed a knowledgeable system based on solubility in water and further divided the soluble DF fraction in two subcategories: oligomers and polymers which possess different rheological properties (Figure 1). Polymeric DFs are mostly viscous whereas oligomeric DFs are not (Tungland 2002). It is generally accepted that oligomers are more easily fermented than polymers or polymeric networks (van de Wiele et al. 2007, Pollet et al. 2012) and that the rate and extent of microbial fermentation of soluble DF is much higher than for insoluble DF (Guillon & Champ 2000). However, these rules of thumb do not always apply. Resistant starch, for example, is insoluble but is also easily fermented.

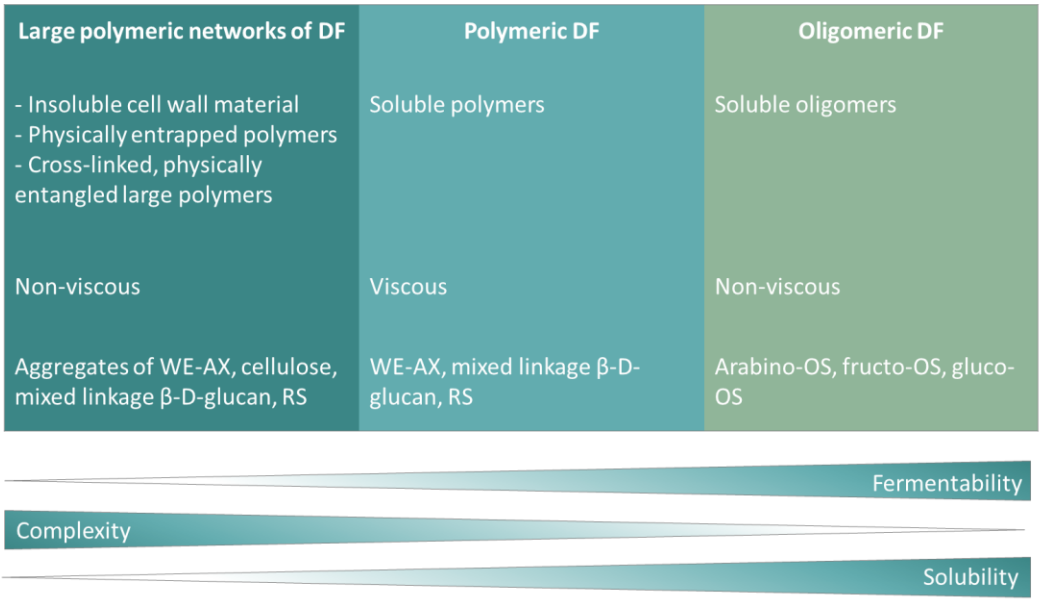


Figure 1 • Classification system based on the physicochemical properties of DF. Three DF categories can be distinguished: large polymeric networks of DF, DF polymers and oligomers. In general, oligomers are more easily fermented while polymers and especially the polymeric networks are considered to be much less fermentable. AX, arabinoxylan; OS, oligosaccharide, RS, resistant starch, WE, water-extractable. Adapted from Verspreet et al. (2016).

1.3 PHYSICOCHEMICAL PROPERTIES

The physicochemical properties of DF highly influence their physiological effects in the gastrointestinal tract. Solubility, viscosity, water holding capacity and adsorption are the main influencing factors.

1.3.1 SOLUBILITY

Dietary fibres are classified as either water soluble or insoluble. Examples of soluble DF are pectic substances, gums and mucilages, whereas cellulose and lignin are insoluble (Elleuch et al. 2011). The solubility of DF depends fully on its structure. In case of insoluble fibres, the molecules are typically arranged in a stable crystalline structure. The backbones of soluble DF on the other hand, show more irregularities and side chains. The presence of charged groups influences solubility as well (Elleuch et al. 2011). Soluble and insoluble DF can be determined as part of standard procedures for the determination of DF (paragraph 1.4, page 7).

1.3.2 VISCOSITY

Viscosity related to DF can be referred to as the ability to increase resistance to flow when mixed with fluids. This thickening results from the physical entanglements of polysaccharides within a solution (Guillon & Champ 2000). Viscosifying properties again depend strongly on the structure of the fibre. Other important parameters are concentration, solvent and temperature (Guillon & Champ 2000). There is a positive, non-linear relationship between the molecular weight of DF in solution and viscosity (Verspreet et al. 2016). This means that soluble fibres that are highly branched or are relatively short chains have low viscosities (Figure 1). The intake of viscous fibres may lower transit times or modify the activities of digestive enzymes, thereby interfering with the digestion and absorption of nutrients (Dikeman & Fahey 2006). Viscosity measurements can be performed using specialized viscometers. In case of wheat bran, water is added to the bran material and centrifuged, whereafter the supernatant is collected for viscosity measurements (Roye et al., unpublished).

1.3.3 WATER HOLDING AND RETAINING CAPACITY

The capacity to hold water is determined mainly by the chemical composition of the component polysaccharides (Guillon & Champ 2000, Elleuch et al. 2011, Jacobs et al. 2015). The origin and the physical characteristics of the fibre are co-determining factors. For example, DF derived from algae present a higher affinity for water than those from fruit. Cereal derivatives showed the lowest affinity for water (Elleuch et al. 2011). The capacity to hold water can be monitored using the Enslin-Neff method where the fibre is presoaked and brought onto a filter paper. By means of a graduated pipet, the amount of released water can be measured (Enslin 1933). When applying external forces, like centrifugation, the water retention capacity can be measured. In this case, only the strongly bound water, e.g. water in nanopores and associated with the fibre material through hydrogen bonding, will remain (Hemdane et al. 2016).

1.3.4 ADSORPTION CAPACITY

The adsorption capacity of DF refers to the ability to associate with water, minerals or endogenic substrates such as bile acids in the gastrointestinal tract. Scavenging of minerals and trace elements by DF impairs their absorption. *In vitro* experiments have shown that this adsorption is mediated by charged polysaccharides (e.g. pectins) and

associated substances (e.g. phytate) in cereal fibres (Guillon & Champ 2000, Greiner & Konietzny 2006, Rebellato et al. 2017). Some DF such as β-glucan, are known to interact with and scavenge bile acids which has cholesterol-lowering effects (Guillon & Champ 2000). Adsorption capacity for a specific compound can be measured by co-incubation of the DF and the compound of interest and measuring the amount of non-bound compound after the incubation time has elapsed, e.g. using spectrophotometrically or pHmetry (Farajzadeh & Monji 2004, Ata et al. 2012).

1.4 ANALYSIS OF DIETARY FIBRE

The measurement of dietary fibres in foods and feeds is a complex issue. Methods can be divided in three categories.

1.4.1 NON-ENZYMATIC-GRAVIMETRIC METHODS FOR DIETARY FIBRE ANALYSIS

These methods were the earliest and include crude fibre, acid detergent and neutral detergent fibre (Englyst et al. 1987). The crude fibre methods provide roughly only cellulose content, while the Van Soest method (1963) measures fibre as the sum of lignin, cellulose and acid insoluble hemicellulose; the neutral detergent method measures fibres as the sum of lignin, cellulose and neutral detergent insoluble hemicellulose (Van Soest 1963, Van Soest & Wine 1967). The methods that fall within this category rely on the successive acid and alkaline digestion to isolate the indigestible fibre fraction. The main shortcoming of this type of methods is that they overlook soluble DF compounds, leading to an underestimation of DF content. The Van Soest method (1963) is still primarily used in veterinary studies to determine crude fibre (Elleuch et al. 2011).

1.4.2 ENZYMATIC-GRAVIMETRIC METHODS FOR DIETARY FIBRE ANALYSIS

Enzymatic-gravimetric methods include enzymatic treatment for removal of starch and proteins, precipitation of soluble DFs using aqueous ethanol, filtration and weighing of the DF residue and correction for protein and ash in the residue (Prosky et al. 1988). Initially these methods underestimated DF contents by overlooking oligosaccharides and resistant starches. Recent protocol adaptations by McCleary et al. (2010) resolved this problem (Figure 2). The authors proposed a method for the measurement of total dietary fibre, including resistant starch and low-molecular-weight nondigestible oligosaccharides of degree of polymerization ≥ 3.

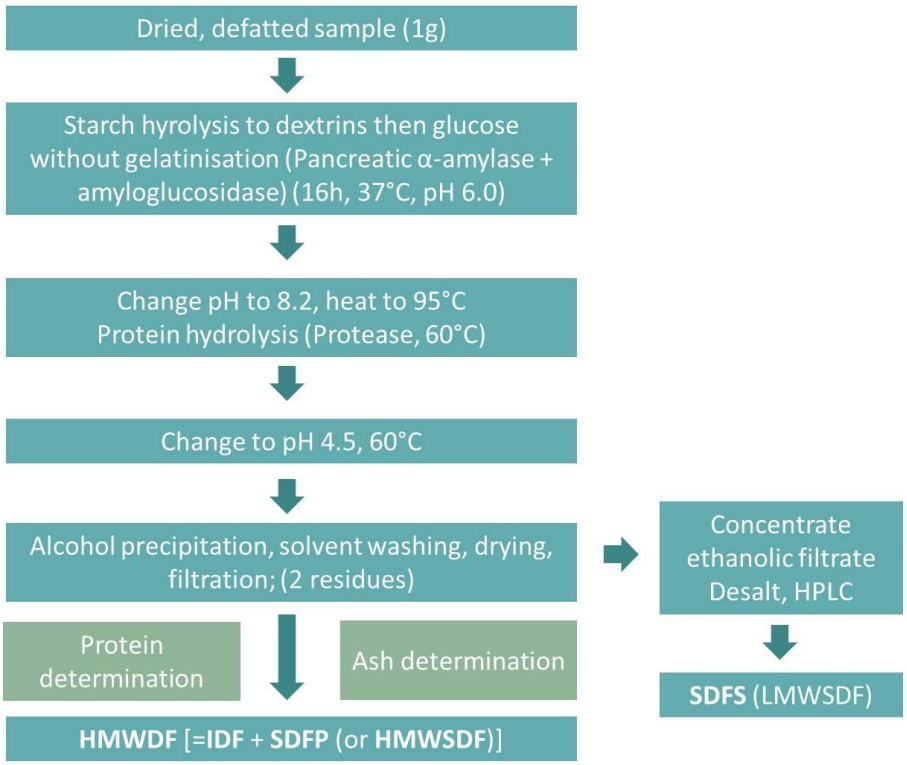


Figure 2 • Analysis of total, soluble and insoluble DF by AOAC method 2009.01. HMWDF: high molecular weight dietary fibre; IDF: dietary fibre insoluble in water; SDFS: dietary fibre soluble in water and soluble in 78% ethanol; SDFP: dietary soluble in water and insoluble in 78% ethanol (McCleary et al. 2010).

1.4.3 ENZYMATIC-CHEMICAL METHODS FOR DIETARY FIBRE ANALYSIS

Like in gravimetric methods, the first step in enzymatic-chemical procedures is the enzymatic removal of starch. To separate soluble DF polysaccharides from low-molecular weight sugars and starch hydrolysis products, precipitation with ethanol 80% (v/v) is used. A frequently applied method, is the Uppsala method (Elleuch et al. 2011). After starch removal, total fibre (both soluble and insoluble) are recovered following precipitation with 80% ethanol and centrifugation. Afterwards, insoluble and soluble fibres are hydrolyzed with sulphuric acid. Neutral sugars can be quantified by gas liquid chromatography, uronic acids by a colorimetric method and Klason lignin can be determined gravimetrically (Theander et al. 1995).

1.5 PROCESSING OF DIETARY FIBRE

One can easily alter the physicochemical properties of DF and enhance functionality by processing. DF can be subjected to enzymatic, chemical, thermal or mechanical treatments. Different processing techniques can be combined as well.

1.5.1 Enzymatic processing

Applying enzymatic treatment may alter the ratio of soluble to insoluble DF. Yacoubi et al. (2016) showed that treatment of wheat with a mixture of endoxylanases and endoglucanases led to an increased amount of water-soluble arabinoxylan and a decreased degree of polymerization of the xylan backbone. The resulting products appeared to be more efficiently fermented into SCFA (Yacoubi et al. 2016). These biological methods are often environmentally friendly and typically there is only little disturbance of the composition and structure of the fibres.

1.5.2 Chemical processing

Chemical methods typically employ acidic and alkaline substances to break down DF. It enables the conversion of some insoluble DF into soluble DF. Another chemical frequently applied is hydrogen peroxide. Treatment of soybean hull with hydrogen peroxide improved the soluble DF content and the resulting material efficiently conjugated bile acids (Feng et al. 2017). However, certain chemical treatments can damage the molecular structure of DF, reducing its physiological activity.

1.5.3 Thermal processing

Thermal treatments can change the ratio between insoluble and soluble fibres, total dietary fibre content, and their physicochemical properties (Caprita & Caprita 2011). Caprita & Caprita (2011) observed that the proportion of soluble DF on total DF increased after heat treatment. The modifications are depended on the type of plant material and on the nature of the treatment (Elleuch et al. 2011, Jacobs et al. 2016b). Both dry and wet heat treatments where shown to increase surface hydrophobicity of DF, lowering the hydration rate (Caprez et al. 1986, Jacobs et al. 2016b). Also, by applying heat, one can easily inhibit all endogenous enzymatic activities. Jacobs et al. (2016b) could, after toasting at 170°C for 30 min, eliminate lipase, α-amylase, endopeptidase, and endoxylanase activities.

1.5.4 Mechanical processing

Mechanical treatments such as grinding can reduce the particle size of DF. Particle size is an important parameter which can significantly influence all physicochemical characteristics described above. Jacobs et al. (2015) showed that, by reducing particle

size of wheat bran, one can easily alter the hydration properties. Measurements of the water holding capacity, swelling capacity, and water retention capacity, as determined by the standard centrifugation method, revealed that smaller particles have a lower water uptake potential (Jacobs et al. 2015). When reducing the particle size of DF, one also increases the specific surface area. By increasing the specific surface area, the surface to which bacteria can attach also increases. This can influence the fermentability of DF because different cell components are made more accessible to microbial enzymes (Stewart & Slavin 2009, Jacobs et al. 2016a).

1.6 BENEFICIAL EFFECTS OF DIETARY FIBRE RELATED TO BROILERS

Fibre intake can have several health-promoting effects which may occur locally and/or systemically. The potential health effect of DF is closely linked with its physicochemical properties, especially solubility. Part of the observed health effects can be attributed to the fermentation of DF in the hindgut and, as stated above, fermentability and solubility show a positive correlation (Guillon & Champ 2000). With regard to broilers, three beneficial effects induced by DF are of main importance, namely beneficial effects (1) on gastrointestinal health status, (2) towards pathogen control and (3) regarding performance.

1.6.1 THE EFFECT OF DIETARY FIBRE ON GASTROINTESTINAL HEALTH

A healthy gastrointestinal tract is, among others, characterized by a balanced intestinal microbiota, where beneficial bacteria outcompete (opportunistic) pathogens, and a mucosal barrier that is strong and reinforced, disabling bacterial translocation. The following paragraphs describe how DF can positively affect these two aspects of gastrointestinal health.

Intestinal health and overall host health are positively correlated. Thus, by improving the health status of the intestine, one can increase significantly overall host well-being (Zhang et al. 2015). There are several indications that gut health can be improved by DF intake. A pivotal role in the underlying mechanism is played by fermentation and the resulting SCFA. By shifting the microbiota composition in the gut, a shift in fermentation products can be induced which can be achieved by consumption of DF (Simpson & Campbell 2015). In rats harboring a human microbiota, the addition of a mix of fructo-oligosaccharides

(FOS) and inulin could induce a bifidogenic effect in the cecum. In addition, in comparison to control-fed rats, higher numbers of lactobacilli and significantly lower numbers of pathogenic clostridia could be noted. In accordance, higher concentrations of butyrate and a reduced pH could be measured in cecum and colon (Kleessen et al. 2001). In another study in rats, the supplementation of AX could induce a 60-fold increase in short chain fatty acids (SCFA)-producing species such as *Roseburia intestinalis* and *Eubacterium rectale*. Again total SCFA were significantly increased in cecal contents (Van den Abbeele et al. 2011). Courtin et al. (2008) observed a bifidogenic effect upon administration of arabinoxylo-oligosaccharides (AXOS) and soluble AX to the diet of broilers (Courtin et al. 2008a). In another broiler study, the in-feed supplementation of xylo-oligosaccharides (XOS) increased cecal levels of members of the butyrate producing *Clostridium* cluster XIVa (De Maesschalck et al. 2015a). So, fermentable DF can either directly stimulate SCFA producing bacteria in the gut or stimulate cross-feeding bacteria, for example lactate producers, which on their turn indirectly stimulate butyrate producers. Several health effects can be attributed, particularly but not exclusively, to butyrate. Butyrate is known to modulate several cellular and metabolic processes such as inflammation in the gut. It counteracts for example the pro-inflammatory cascade induced by NFκB (Inan et al. 2000), inhibits the production of IFNγ (Klampfer et al. 2003) and upregulates PPARγ (Wachtershauser et al. 2000). In addition butyrate has been shown to modulate oxidative stress (Hamer et al. 2008) and reinforce the mucosal barrier (Peng et al. 2007).

The mucosal barrier in the gut is an important defense mechanism against pathogens. It also delivers immunoregulatory signals to the underlying epithelium. SCFA are known to enhance the development and the conservation of this barrier by inducing epithelial cell proliferation and differentiation (Guilloteau et al. 2010). This can be partly explained by the fact that butyrate is the preferred energy source of epithelial cells in the distal segments of the gut (Sakata & von Engelhardt 1983). It is both passively and actively taken up by epithelial cells and metabolized via fatty acid oxidation (De Preter et al. 2011). In addition to the direct proliferative effect of SCFA on epithelial cells of the hindgut, another trophic effect of SCFA can be observed related to glucagon-like peptide 2 (GLP-2) secretion. GLP-2 is a 33-amino acid long peptide produced by the enteroendocrine L-cells in the gut (Burrin et al. 2001). In chickens, these L-cells are strategically positioned in the terminal jejunum and

ileum, where they can easily monitor SCFA release (Tappenden & McBurney 1998). The GLP-2R receptor is distributed throughout the intestine and is very specifically bound by its ligand (Munroe et al. 1999). GLP-2 secretion induces increased tissue mass and mucosal thickness in the gut. Microscopically increased villus length and crypt depth are observed, leading to a more efficient absorption process (Drucker et al. 1996). Thulesen et al. (1999) could link the intake of DF with increased GLP-2 plasma levels in a diabetic rat model. They propose a mechanism where DF in the hindgut is fermented by the resident microbiota into SCFA. These SCFA would then induce the secretion of the intestinotrophic GLP-2, which positively affected the mucosal barrier (Thulesen et al. 1999). Next to the proliferative effects of SCFA on the intestinal epithelium, butyrate is known to stimulate mucin production through the upregulation of *MUC2* gene expression, encoding a glycosylated mucin (Jiang et al. 2013). In addition it enhances the expression of tight junction proteins, such as claudin-1, reinforcing the epithelial barrier (Wang, H. et al. 2012).

1.6.2 THE EFFECT OF DIETARY FIBRE ON BROILER PERFORMANCE

In the past, DF was considered to be a diluent in animal feed which was supposed to negatively impact feed intake and nutrient digestibility. However, more recently, research has pointed out that the inclusion of moderate amounts of DF can significantly ameliorate the development of digestive organs (Hetland et al. 2003, Hetland et al. 2005, Gonzalez-Alvarado et al. 2007), improve nutrient digestibility (Amerah et al. 2009, Jimenez-Moreno et al. 2009) and growth performance (Amerah et al. 2009, Jimenez-Moreno et al. 2009, Gonzalez-Alvarado et al. 2010). However, the effects of DF supplementation depend strongly on the physicochemical properties of the fibre (Saki et al. 2011, Mateos et al. 2012), the inclusion level, the physical structure of the fibre and the age and breed of the birds (Mateos et al. 2012). In practice, particle size and solubility and the degree of lignification are key characteristics affecting broiler performance. This is because DF influences passage rate in the upper intestine and fermentation efficiency in the lower part (Saki et al. 2011).

Crude DF mixtures such as oat, corn, soy or pea hulls frequently are supplemented to broiler feeds to enhance performance. Increasing the insoluble DF fraction of the diet often results in an increased gizzard weight (Hetland et al. 2005, Gonzalez-Alvarado et al. 2007, Jimenez-Moreno et al. 2010) and improved performance (Gonzalez-Alvarado et al. 2007). A more developed

gizzard produces and secretes more hydrogen chloride and endogenous enzymes, such as pepsin, that enhance digestion of feed particles (Mateos et al. 2012). It was stated that feed particles should be larger than one mm in order to stimulate gizzard development (Svihus 2011). This statement was supported by trials in which microcrystalline cellulose (geometric mean diameter of 73 µm) was added to the diet of broilers. Inclusion of these small particle size DF to a low-fibre diet led to a less developed GIT and higher gizzard pH compared to the supplementation of larger DF particles (Jimenez-Moreno et al. 2010).

In addition to the above described crude mixtures of uncharacterized DF, purified oligosaccharides are often applied. However, outcomes are not always consistent. Many oligosaccharides that are able to increase performance are considered to exert prebiotic effects. As a consequence, one often observes beneficial shifts in microbiota composition along with an enhanced performance (Courtin et al. 2008a, De Maesschalck et al. 2015a, Yang et al. 2016). Compounds such as AXOS, XOS, fructo-oligosaccharides (FOS) and inulin induce increases in numbers of *Lactobacillus* and *Bifidobacterium* species, but also members of the butyrate producing families *Lachnospiraceae* and *Ruminococcaceae* (Kaplan & Hutkins 2000, Biggs et al. 2007, Courtin et al. 2008b, Scott et al. 2014, De Maesschalck et al. 2015a). The possible link between an increase in abundance of these organisms and improved performance is the production of SCFA. As described above, these SCFA have several beneficial effects on intestinal health and this might, at least partly, explain the observed improved performance.

(A)XOS, which are degradation products from xylan, have been shown to increase broiler performance significantly when supplemented to a wheat-rye based diet. The improved performance was accompanied by an increase in *Lactobacillus* species in the colon and *Ruminococcaceae* and *Lachnospiraceae* species in cecal contents (De Maesschalck et al. 2015a). With regard to performance, studies using FOS and inulin give contradicting results ranging from improved performance, poor performance to no effect at all (Biggs et al. 2007, Williams et al. 2008, Geier et al. 2009, Kim et al. 2011, Ao & Choct 2013). Yet, often, an increase in beneficial bacterial groups, such as lactobacilli, can be observed (Geier et al. 2009, Kim et al. 2011). By supplementing 0.1% β-glucan to a maize-soybean diet, Cho et al. (2013) could increase body weight gain significantly compared to control-fed chickens (Cho et al. 2013).

When overlooking these studies, one clearly notices that the outcome of this kind of trials is extremely variable but birds require a minimum and maximum amount of fibre in the diet for optimal performance. The amount of DF required depends, as mentioned earlier, on its physicochemical properties, being solubility, lignin content and particle size (Mateos et al. 2012) but also on the nature of the basal diet (Jimenez-Moreno et al. 2009).

Table 1
Overview of studies evaluating in-feed supplementation of prebiotics on broiler performance

Compound	Concentration	Performance	Basal diet	Reference
XOS	0.2% (starter) 0.5% (grower/finisher)	↓ FCR ratio ↓ FCR ratio	Wheat/Rye	(De Maesschalck et al. 2015b)
Inulin	0.4% 0.8%	↑ ME ↓ ME	Corn/Soy	(Biggs et al. 2007)
Oligofructose	0.4% 0.8%	↑ ME	Corn/Soy	(Biggs et al. 2007)
Short chain FOS	0.4% 0.8%	↑ ME ↓ ME, ↓ AA digestibility	Corn/Soy	(Biggs et al. 2007)
MOS	0.4% 0.8%	↑ ME, ↑ AA digestibility	Corn/Soy	(Biggs et al. 2007)
TOS	0.4% 0.8%	↑ ME ↓ AA digestibility	Corn/Soy	(Biggs et al. 2007)
FOS	0.06%	↓ Daily weight gain, ↑ FCR	Wheat/Soy	(Williams et al. 2008)
MOS	0.5%		Wheat	(Geier et al. 2009)
FOS	0.5%		Wheat	(Geier et al. 2009)
FOS	0.25% 0.5%	↑ Body weight gain	Corn/Soy	(Kim et al. 2011)
MOS	0.025% 0.05%	↑ Body weight gain ↑ Body weight gain	Corn/Soy	(Kim et al. 2011)
MOS	0.1% (starter) 0.05% (grower/finisher)	↑ Body weight gain ↓ FCR ratio ↑ Body weight gain	Sorgum/Soy	(Ao & Choct 2013)
FOS	0.1% (starter) 0.05% (grower/finisher)	↑ Body weight gain ↓ FCR ratio ↑ Body weight gain	Sorgum/Soy	(Ao & Choct 2013)
B-glucan	0.1%	↑ Body weight gain	Corn/Soy	(Cho et al. 2013)

FCR: feed conversion rate; ME: metabolizable energy; AA: amino acid

1.6.3 PATHOGEN CONTROL – THE EXAMPLE OF *SALMONELLA*

In the following paragraphs a confined overview will be given on the issue of *Salmonella*. It is not the purpose of this thesis to address pathogenicity, invasion mechanisms and control measures in great detail. The following elaborate reviews can be consulted for that matter: Dunkley et al. (2009), EFSA (2015), LaRock et al. (2015).

Salmonella is a zoonotic agent of global importance. Despite the fact that from 2008 to 2014, a significant decrease in the annual total number of *Salmonella* outbreaks was observed, still 20% of all foodborne disease in European citizens was caused by *Salmonella* in 2014 (EFSA 2015). Most human salmonellosis cases are caused by the consumption of contaminated food. In case of poultry derived products one considers eggs and broiler meat as the most important sources of *Salmonella*. Especially minced meat and meat preparations from poultry show high levels of non-compliance with EU *Salmonella* criteria (EFSA 2015). In 2014 the most important causative serovar was *Salmonella* Enteritidis accounting for 44.4% of the cases (EFSA 2015).

FACTORS AFFECTING SUSCEPTIBILITY OF POULTRY TO *SALMONELLA*

Chickens can be contaminated by *Salmonella* either vertically or horizontally (Liljebjelke et al. 2005). The vertical transmission route implies the transfer from parent to offspring and is rather straightforward to control. Horizontal transmission, however, is less easy to control since *Salmonella* can potentially enter at numerous points into the integrated poultry production system (Liljebjelke et al. 2005, Vandeplas et al. 2010). Examples of potential sources of contamination are feed and drinking water (Marin et al. 2011), rodent or insect infestations (Meerburg & Kijlstra 2007), dust (Marin et al. 2011), inadequate cleaning and decontamination of the rearing houses (Marin et al. 2011) and insufficient hygienic measures of staff (Quast et al. 2013).

Age and diet are important factors that affect the susceptibility of poultry to *Salmonella*. Young animals are extremely susceptible to enteric pathogens because they lack a well-developed gut microbiota. Due to the absence of direct contact between the parental flock and hatchling, the transfer of the commensal microbiota is hampered (Van

Immerseel et al. 2009, Vandeplas et al. 2010). A dense resident microbial community in the gut is essential for many host physiological processes that include enhancement of the intestinal epithelial barrier, development of the immune system and acquisition of nutrients. A major function of the commensal microbiota is protection against colonization by pathogens (Kamada et al. 2013). Both direct and indirect protection mechanism have been described. Direct mechanisms include the production of specific metabolites such as bacteriocins (Wang, Q. et al. 2012) and SCFA (Gantois et al. 2006) that negatively affect the pathogen. Indirect mechanism relate to the clearance of pathogens by the stimulation of the host immune response (Abt & Artis 2013).

Feed has also been shown to affect the susceptibility to *Salmonella* infections. Teirlynck et al. (2009) showed that the cereal type influences the susceptibility of broilers to *Salmonella* infections. A higher colonization rate of ceca, spleen and liver was observed when broilers were fed a wheat-rye diet compared to a corn-based diet (Teirlynck et al. 2009b). The experimental wheat-rye diet inflicts mucosal damage and a significant change in microbiota composition which may favor the colonization of *Salmonella* (Teirlynck et al. 2009a). Also the structure of the feed seems to influence *Salmonella* susceptibility. The inclusion of whole wheat in the diet enables a better development of the gizzard and the accompanying decrease in pH evokes a reduction in *Salmonella* counts (Klasing 1998, Bjerrum et al. 2005). Feed form and particle size have also been shown to affect *Salmonella* colonization. Pelleted diets are linked with higher *Salmonella* counts when compared to mash diets (Huang et al. 2006).

PATHOGENESIS

Chickens are almost always infected by the uptake of bacteria from the environment via oral ingestion (LaRock et al. 2015). When entering the GIT, *Salmonella* bacteria need to cope with the acidic environment of the stomach. They succeed by expressing several acid shock proteins that offer resistance to the low pH (Foster 1991). Next, the bacteria migrate towards the epithelial lining of the gastrointestinal tract and make contact with the enterocytes. The predominant sites of *Salmonella* colonization are the cecal pouches. Migration and attachment is facilitated by flagellae and fimbriae (Darwin & Miller 1999, van Asten & van Dijk 2005). Upon attachment *Salmonella* expresses a type three secretion system

(T3SS), a multiprotein effector complex which is encoded on *Salmonella* Pathogenicity Island 1 (SPI-1) (Foley et al. 2008, Winnen et al. 2008). This genomic region contains several genes related to the initial invasion as well as regulatory effectors that orchestrate the process. The T3SS spans the outer and inner membrane of the bacterial cell wall and its main function is the injection of effector proteins in the epithelial cell (Burkinshaw & Strynadka 2014). The effector proteins interact with the actin cytoskeleton of the epithelial cell and induce remodeling of the actin filaments (Raffatellu et al. 2005, Velge et al. 2012). These rearrangements cause the outward extension of the host cell, also called membrane ruffling, which induces the engulfment of the *Salmonella* bacteria. The bacteria become internalized in the so called *Salmonella* containing vacuole (SCV) which protects it from the hostile environment of the cell cytoplasm (Lostroh & Lee 2001). This vesicle not only shields *Salmonella* from host cell defenses, it also enables proliferation. A fraction of the SCVs will transcytose to the basolateral membrane of the epithelial cell where the bacteria are taken up by phagocytes (Fabrega & Vila 2013). In addition, it has been reported that dendritic cells can directly take up *Salmonella* bacteria by sampling the intestinal lumen (Rescigno et al. 2001).

REGULATION OF INVASION

As mentioned before, the expression of invasion related genes is tightly regulated by both regulatory and environmental factors, the latter including osmolarity, oxygen concentration and pH (Galan & Curtiss 1990, Bajaj et al. 1996). *HilA* is the main transcriptional regulator of the SPI-1 cluster of invasion genes in *Salmonella* (Fahlen et al. 2000). Its transcription is controlled by several other regulatory proteins such as *HilC* and *HilD*. These regulatory cascades are initiated or blocked by means of environmental signals. The expression of SPI-1 is induced when oxygen levels are low, osmolarity is high and at neutral to slightly basic pH; conditions that prevail in the GIT. Furthermore it is known that acetate can induce expression of SPI-1 genes, while butyrate and propionate have the opposite effect by downregulating the expression of important regulators such as *HilA* (Lawhon et al. 2002, Gantois et al. 2006, Van Immerseel et al. 2006).

NUTRITIONAL TOOLS TO CONTROL *SALMONELLA*

As stated above, feed composition and formulation can significantly affect the susceptibility to *Salmonella*. In addition, adding specific supplements to the feed and drinking water of broilers can aid in the control of *Salmonella*. Organic acids are a frequently applied example. SCFA such as butyrate, and medium chain fatty acids (MCFA) such as caproic acid, fall under this category and have proven their worth already (Van Immerseel et al. 2004, Van Immerseel et al. 2006, Van Immerseel et al. 2009). Other important feed additives are prebiotics. Prebiotics are non-digestible food or feed components that induce beneficial physiological effects on host health by stimulating growth and/or activities of beneficial bacteria in the intestine (Gibson et al. 2004, Bindels et al. 2015). Since these components are non-digestible, they end up in the hindgut where they are (partly) fermented by the resident microbiota. So far, a wide variety of carbohydrates such as FOS, inulin, lactulose, galacto-oligosaccharides (GOS), XOS and AXOS have been proposed as prebiotic candidates (Rastall & Maitin 2002, Damen et al. 2011, Bindels et al. 2015). Several studies have evaluated the use of these DF in *Salmonella* infection models (Bailey et al. 1991, Tellez et al. 1993, Chambers et al. 1997, Spring et al. 2000a, Eeckhaut et al. 2008). Eeckhaut et al. (2008) demonstrated that the addition of 0.4% of wheat bran derived AXOS could reduce colonization and shedding of *Salmonella* in broilers. An explanation may be found in the observations made by Courtin et al. (2008), who have shown that AXOS induce a bifidogenic effect when administered in the feed of broilers (Courtin et al. 2008b). By increasing competitive pressure, these bacteria can prevent pathogen colonization (Derrien & Vlieg 2015). Fernandez et al. (2002) observed also an increase in *Bifidobacterium* and *Lactobacillus* species when supplementing mannan-oligosaccharides (MOS) to the diet of *Salmonella* challenged broilers. The overall numbers of *Enterobacteriaceae* decreased, as well as the *Salmonella* counts (Fernandez et al. 2002). Chambers and others (1997) evaluated the administration of 5% FOS, 5% lactulose, and 5% lactosucrose in a *Salmonella* Typhimurium infection model. All three components induced reductions in cecal counts (Chambers et al. 1997). The supplementation of 0.4% MOS could induce a log reduction in cecal counts of *Salmonella* (Spring et al. 2000a). Bailey et al. (1991) noted that the number of chickens colonized with *Salmonella* could be reduced when administering 0.75% of FOS in their diet (Bailey et al. 1991) while Tellez et al. (1993) used 10% of lactose to

reduce the total number of *Salmonella* positive organ samples in an Enteritidis infection model (Tellez et al. 1993). These studies all together indicate promising effects for the use of purified, fermentable DF in the control of *Salmonella* infections.

Table 2
Confined overview of prebiotic intervention studies in *Salmonella* infection trials.

Compound	Concentration	Reference
AXOS	0.4%	(Eeckhaut et al. 2008)
MOS	2.5%	(Fernandez et al. 2002)
FOS	5%	(Chambers et al. 1997)
Lactulose	5%	(Chambers et al. 1997)
Lactosucrose	5%	(Chambers et al. 1997)
MOS	0.4%	(Spring et al. 2000b)
FOS	0.75%	(Bailey et al. 1991)
Lactose	10%	(Tellez et al. 1993)

2. WHEAT BRAN: A HIGHLY CONCENTRATED SOURCE OF DIETARY FIBRE

Wheat bran is a relatively cheap byproduct of the milling of wheat into flour and is highly concentrated in both soluble and insoluble DF. It is a substrate which can be easily technically modified. By means of technical modification, one can improve and optimize the properties of the constituting DF fractions, yielding an optimal impact on host health.

2.1 DIETARY FIBRE COMPOSITION OF WHEAT BRAN

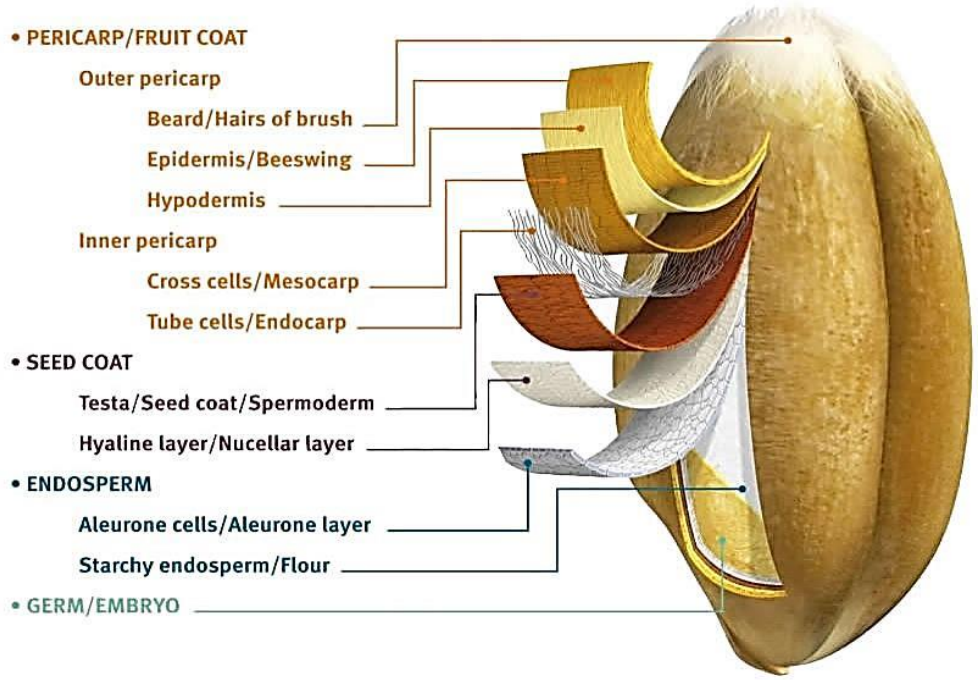


Figure 3 • Layers of wheat. The bran fraction comprises aleurone, nucellar epidermis (or hyaline layer), seed coat, inner pericarp and the outer pericarp layers. Obtained from Surget & Barron (2005).

Wheat (*Triticum aestivum*) is an important staple food in many countries. One can distinguish three main structures in a wheat kernel: the germ or embryo, the endosperm, and the surrounding bran (Figure 3; Figure 4) (Panato et al. 2017). The cell walls in these tissues have different properties and compositions (Panato et al. 2017). Cell walls of bran material are typically thick, hydrophobic and composed of cellulose, complex xylans and lignin. The cell walls in endosperm, on the other hand, are thin, hydrophyllic and consist mainly of arabinoxylan and mixed-linked β-glucans (Saulnier et al. 2007).

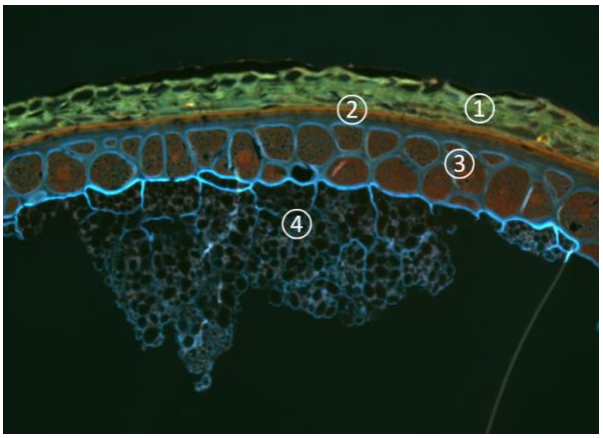


Figure 4 • Cross-section of wheat bran stained with acid fuchsin and calcofluor. Acid fuchsin stains proteins red and calcofluor stains β-glucan blue in epifluorescent light (excitation 400 to 410nm, emission >455nm). The pericarp is not stained, but can be detected due to autofluorescence. ① pericarp, ② seed coat & nucellar epidermis, ③ aleurone layer, ④ starchy endosperm. Adapted from Hemdane et al. (2016).

The bran fraction is composed of several layers: aleurone, nucellar epidermis (or hyaline layer), seed coat, inner pericarp and the outer pericarp (Figure 3; Figure 4) (Surget & Barron 2005, Hemdane et al. 2016, Panato et al. 2017). These layers constitute up to 14-16% of the wheat kernel (Maes & Delcour 2001, Akhtar et al. 2012, Stevenson et al. 2012).

During the milling of wheat into flour, the starchy endosperm is separated from the bran material and embryo. The endosperm fraction is further ground to white flour while the bran material is a by-product. Irrespective of the milling process, remnants of starchy endosperm will always be attached to the bran fraction. On total bran basis the pericarp makes up 6-23% of the bran, seed coat and nuclear epidermis 6-30%, aleurone 33-52% and starchy endosperm 9 to 35% (Figure 4) (Maes & Delcour 2001, 2002).

Table 3
Chemical composition and arabinose-xylose ratio of regular wheat bran, pericarp and aleurone, expressed as percentage range. n.a. not applicable. Obtained from Hemdane et al. (2016).

	Regular bran	Pericarp	Aleurone
Arabinoxylan	17-33	42-46	20-46
A/X ratio	0.46-0.51	1.06-1.15	0.36-0.39
Cellulose	9-14	22-40	1-3
Fructan	3-4	n.a.	5
β-D-glucan	1-3	3-9	5-16
Starch	6-30	0-6	0-11
Proteins	14-26	6-10	21-30
Lipids	3-4	0-1	4-9
Ash	5-7	2-7	7-12

Wheat bran is a highly concentrated source of both fermentable and non-fermentable DF (Leitch et al. 2007). Chemically one can distinguish the following components: AX, cellulose, fructan, and mixed linked β-D-glucan. Next to these non-starch carbohydrates, bran also contains starch, protein, lipids, lignin, minerals, phytic acid and phenolic acids (Figure 5; Table 3). These components are not evenly distributed over the bran but are concentrated in specific tissue layers (Table 3). The pericarp for example, is rich in insoluble DF such as cellulose, lignin and arabinoxylans with a high degree of substitution. The aleurone layer on the other hand contains relatively more soluble DF such as β-glucans and arabinoxylans with a low degree of substitution (Saulnier et al. 2007). Moreover, the aleurone is known to be particularly rich in nutrients when compared to pericarp. It contains, among others, relatively high concentrations of essential amino acids, vitamins, antioxidants, and minerals (Table 3) (Stevenson et al. 2012).

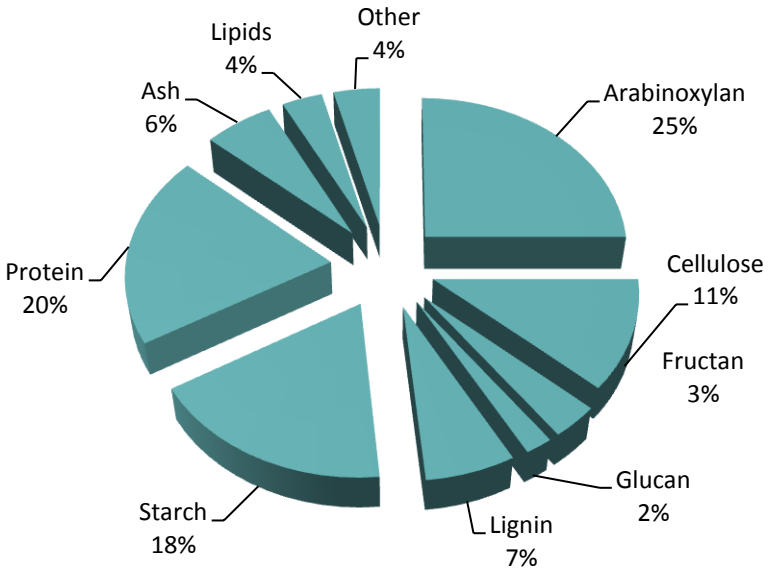


Figure 5 • Main wheat bran constituents. Adapted from Hemdane et al. (2016).

2.1.1 ARABINOXYLAN

Most of the constituting fibres in the cell walls of wheat are AXs. The linear xylose backbone of AX consists of β-(1,4)-D-xylopyranosyl units which may be substituted with α-L-arabinofuranosyl residues on the C(O)-2 and/or C(O)-3 position (Dornez et al. 2009). The C(O)-5 position of the arabinose units can sometimes be ester-linked to ferulic acid (Table 4, Figure 6) (Cleemput et al. 1993). The structure of AX differs between histological layers: wheat bran AX can be substituted with additional glucuronic acid and galactose residues, while this is not the case for AX from wheat endosperm (Saulnier et al. 2007).

AXs can be characterized based on their degree of polymerization (i.e. the average number of xylose units per molecule of AX) and their average degree of substitution (i.e. the number of arabinose substituents on the xylose backbone). Both parameters are important determinants for several physicochemical properties of AX (e.g. solubility). AXs derived from wheat show on average a lower degree of substitution compared to sorghum and rice, in other words, they contain higher proportions of unsubstituted xyloses or lower levels of monosubstituted xylose residues (Maes & Delcour 2002, Stevenson et al. 2012). Nonetheless, the highest levels of double substituted xylose residues can be found in AX from wheat pericarp (Maes & Delcour 2002). Fractions of AX with a high average degree of substitution are more difficult to degrade than AX with a low or intermediate substitution degree (Damen et al. 2011). The A/X ratio also differs between histological layers; pericarp is characterized by a very high A/X ratio of 1.14, while for aleurone this ratio is 0.4 (Saulnier et al. 2007). Even within the different pericarp tissues, the degree of substitution fluctuates considerably (Saulnier et al. 2007).

Two classes of AX exist in wheat: water-extractable (WE-AX) and water-unextractable AX

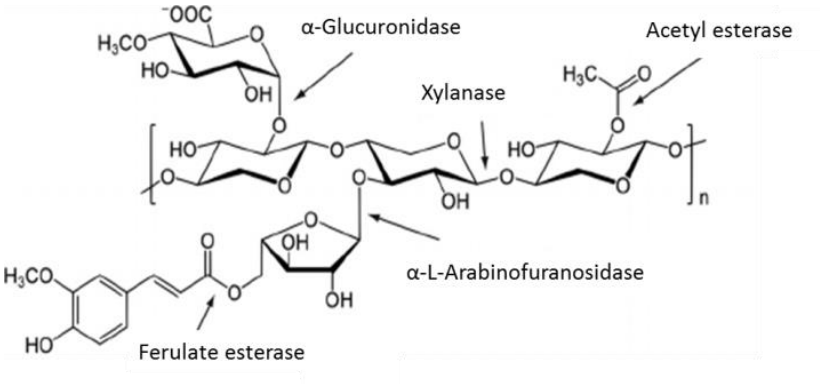


Figure 6 • Structure of arabinoxylan and the cleavage sites of xylanolytic enzymes involved in its degradation. Adapted from Rogowski et al. (2014).

(WU-AX). The water extractability of AX is correlated with substitution patterns and physical entanglement. In general, this means that the WE-AX fraction is loosely bound to the cell wall surface while the WU-AX is retained in the cell wall by covalent and non-covalent bonding with other AXs, proteins, lignin and cellulose, which makes it less accessible for enzymes (Saulnier et al. 2007, Flint et al. 2012, Knudsen 2014). When comparing with other cereals, aside from rye, wheat contains the highest amount of WE-AX (Nordlund et al. 2012).

The intestinal microbiota possesses an extensive armory of AX degrading enzymes. The AX molecule is broken down by endo-1,4-β-xylanases, α-L-arabinofuranosidases, β-xylosidases, α-glucuronidases and ferulic acid esterases (Figure 6) (Hughes et al. 2007, Vardakou et al. 2007, Grootaert et al. 2009, Rogowski et al. 2014). Beta-xylosidases release xylose monomers from the non-reducing ends of AX, while xylanases cleave the xylose backbone randomly. Arabinose is removed from the xylose backbone by α-L-arabinofuranosidases (Biely et al. 1985, Henrissat & Bairoch 1996). Bacterial species belonging to the genera *Lactobacillus*, *Prevotella*, *Bacteroides* and *Clostridium* are often specialized in degrading complex carbohydrates such as AX (Cotta 1993, Cotta & Zeltwanger 1995, Miyazaki et al. 1997, Kobayashi et al. 1998, Erlandson et al. 2001, Crittenden et al. 2002, Hayashi et al. 2005). In addition, several studies have shown that certain *Bifidobacterium* species are able to dismantle AX by removing arabinose residues from the backbone using arabinofuranosidases (Table 4) (Crittenden et al. 2002, van den Broek et al. 2005, Riviere et al. 2014, Truchado et al. 2015).

Table 4
Chemical structures and respective degradation enzymes of the most important DF occurring in wheat bran. Adapted from Hamaker & Tuncil (2014).

Dietary fibre	Chemical structure	Degradation enzymes[a]	CAZymes families	References
Cellulose	linear β-(1,4) Glu units	Endoglucanase, Cellobiohydrolases, β-glucosidases	GH1, GH3, GH5, GH7, GH8, GH9, GH44, GH48, GH51, GH74	(Himmel 2007, Kumar et al. 2008, Liu et al. 2009, Lombard et al. 2014)
Arabinoxylan	β-(1,4) Xyl units as the backbone with side chains of Ara units linked via α-(1,2), α-(1,3) and α-(1,5). Gal, GluA or FerA unit may be present in branch point	Xylanase, α-glucuronidase, α-L-arabinofuranosidase, β-xylosidase, feruloyl esterase, acetyl xylan esterase	GH3, GH5, GH7, GH8, GH10, GH11, GH39, GH43, GH51, GH52, GH54, GH62, GH67, GH115, CE1, CE2, CE4, CE6, CE7	(Hughes et al. 2007, Vardakou et al. 2007, Grootaert et al. 2009, Lombard et al. 2014)
β-glucan	Repeating linear polymer of β-(1,4) Glu alternated with β-(1,3) Glu units	Lichinase, β-glucan endohydrolase, endo-(1,4) β-glucanase	GH5, GH6, GH8, GH9, GH10, GH12, GH16, GH26, GH44, GH45, GH48, GH51	(Colleoni-Sirghie et al. 2003, Beckmann et al. 2006, Lazaridou & Biliaderis 2007, Lombard et al. 2014)
Fructan	β-(2,1) Fru β-(2,6) Fru	Inulinase, β-(2,1) fructanlyase	GH32, GH91	(Singh & Gill 2006, Kango & Jain 2011, Flint et al. 2012, Lombard et al. 2014)

Glu: glucose, Xyl: xylose, Ara: arabinose, Gal: galactose, GluA: glucuronic acid, FerA: ferulic acid
[a]Enzymes required for the complete degradation of the corresponding DF structure

2.1.2 B-GLUCAN

In wheat, β-glucans can be found mainly in the subaleurone layer and to a lower extent in the endosperm (Lazaridou & Biliaderis 2007). They are homopolysaccharides of D-glucopyranosyl

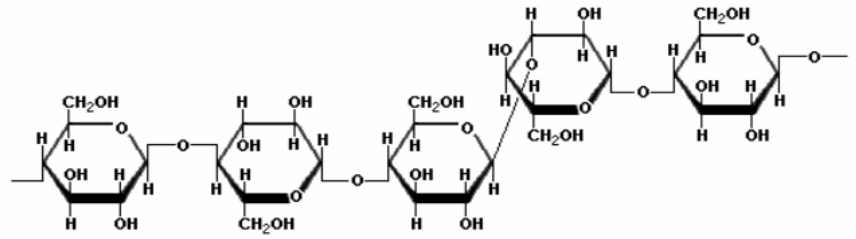


Figure 7 • Structure of mixed linked β-glucan. Obtained from Havrlentova et al. (2011).

residues linked by alternating β-(1→3) and β-(1→4) linkages. Mostly blocks of consecutive (1→4)-linked residues (i.e. cellulose oligomers) are found, separated by a single (1→3) linkage (Table 4, Figure 7) (Lattimer & Haub 2010, Havrlentova et al. 2011, Hamaker & Tuncil 2014). The cellulose segments are in general restricted to three or four constituting glucose residues (Johansson et al. 2000, Colleoni-Sirghie et al. 2003, Lazaridou & Biliaderis 2007, Wood 2010). Depending on the cereal, the ratio between these tri- and tetramers can differ significantly, as well as the ratio of β-(1→3) and β-(1→4) linkages (Lazaridou & Biliaderis 2007, Wood 2010). Due to the presence of the (1→3) linkages, the rigid structure is interrupted on regular basis and this contributes to the solubility of β-glucans in water (Colleoni-Sirghie et al. 2003, Lazaridou & Biliaderis 2007, Izydorczyk & Dexter 2008, Wood 2010).

Bacterial degradation of β-glucans is accomplished by means of 1,3-1,4-β endoglucanases (Table 4) (Beckmann et al. 2006). These enzymes specifically cleave the (1→4) glycosidic linkage and the major hydrolysis products are cellobiosyl-(1,3)-D-glucose and cellotriosyl-(1,3)-D-glucose (Colleoni-Sirghie et al. 2003, Lazaridou & Biliaderis 2007). Beckmann et al. (2006) isolated and identified mixed linked β-glucan degrading bacteria in the intestine of broilers. They could be assigned to the genera *Streptococcus*, *Enterococcus*, *Bacteroides* and *Clostridium* (Beckmann et al. 2006).

2.1.3 CELLULOSE

Cellulose is a linear chain of β(1,4)-linked glucopyranosyl units which is a major structural component in all plant cell walls (Table 4, Figure 8) (Osullivan 1997, Pinkert et al. 2009, Lattimer & Haub 2010, Ciolacu et al. 2011). Cellulose is present in the cell wall of all cell tissues but in wheat for

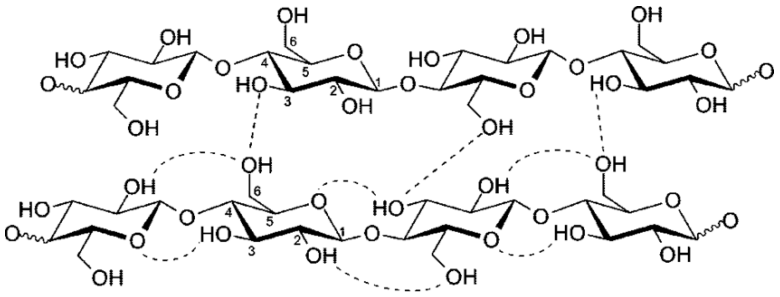


Figure 8 • Chemical structure of cellulose. Intra- and intermolecular hydrogen bonds are indicated with dotted lines. Obtained from Pinkert et al. (2009).

example, concentrations are higher in pericarp than in endosperm and embryo (Knudsen 2014). Hydrogen bonding between the hydrogen of the C-3 OH with the ring oxygen of the adjacent glucose monomer, stabilizes the chain intramolecularly while intermolecular connections are made between O-6 of a glucose residue in one chain and O-1 in another (Osullivan 1997, Flint et al. 2012, Knudsen 2014). Cellulose chains aggregate laterally in microfibrils which are embedded in a matrix of hemicelluloses (AX and β-glucan), pectins and lignin in the plant cell wall (Figure 10) (Flint et al. 2012, Medie et al. 2012).

Two types of cellulose can be distinguished, crystalline and amorphous cellulose. The crystalline form is very rich in the above described hydrogen bonds, causing it to be insoluble in water. The amorphous form is characterized by less intra- and intermolecular hydrogen bonding (Osullivan 1997, Lattimer & Haub 2010, Flint et al. 2012). Highly crystalline cellulose is also recalcitrant to enzymatic degradation whereas the amorphous form is more easily accessible (Ciolacu et al. 2011).

The enzymatic hydrolysis of cellulose is a complex process and is carried out in the intestine by specialized cellulolytic microorganisms (Ciolacu et al. 2011). Three different cellulases work synergistically in a cellulase complex to degrade the cellulose chain: (1) endoglucanases randomly hydrolyze β-1,4-glycosidic bonds at internal positions (Liu et al. 2009), (2) exoglucanases release soluble glucose or cellobiose at the chain-termini, and (3) β-glucosidases, which hydrolyze cellobiose to glucose (Table 4) (Himmel 2007, Kumar et al. 2008). Hemicelluloses, such as AX, and the aromatic polymer lignin, interact with the cellulose fibrils, creating an extremely rigid structure. Therefore, complete and rapid hydrolysis of cellulose requires the collaboration of both xylanolytic and cellulolytic bacteria (Beguin & Aubert 1994). Human fecal bacteria encoding cellulases belong to the genera *Clostridium*,

Eubacterium, *Enterococcus, Bacteroides* and *Ruminococcus*. (Medie et al. 2012, Hamaker & Tuncil 2014). A recent study indicates that *Alistipes* species contribute to the degradation of cellulose in the chicken intestine as well (De Maesschalck et al., unpublished).

2.1.4 FRUCTAN

Fructans are carbohydrates that are composed mainly or even exclusively of fructose. Usually none or only one glucose unit is present (Lewis 1993). Fructan molecules can be classified based on their core molecule and the type of fructose-fructosyl linkages (either β-2,1 or β-2,6). This core consists of a fructose and sucrose, and depending on the site of attachment of the fructose residue, one distinguishes either 1-kestotriose, 6-kestotriose, or 6G-kestotriose (Figure 9) (Verspreet et al. 2015a). These core molecules form the basis of five different fructan types (Figure 9):

1. Inulin-type fructans are the best known type of fructans. They contain a 1-kestotriose core and have a linair structure of β-2,1 linked fructosyl units,
2. Levan-type fructans contain a core of 6-kestotriose and are mainly composed of β-2,6 linked fructosyl units,
3. Graminans have both β-2,1 and β-2,6 fructosyl linkages and a complex, branched structure,
4. Neo-inulin type fructans are built from 6G-kestotriose and characterized by β-2,1 linkages, while
5. Neo-Levan-type fructans contain predominatly β-2,6 linked fructosyl units.

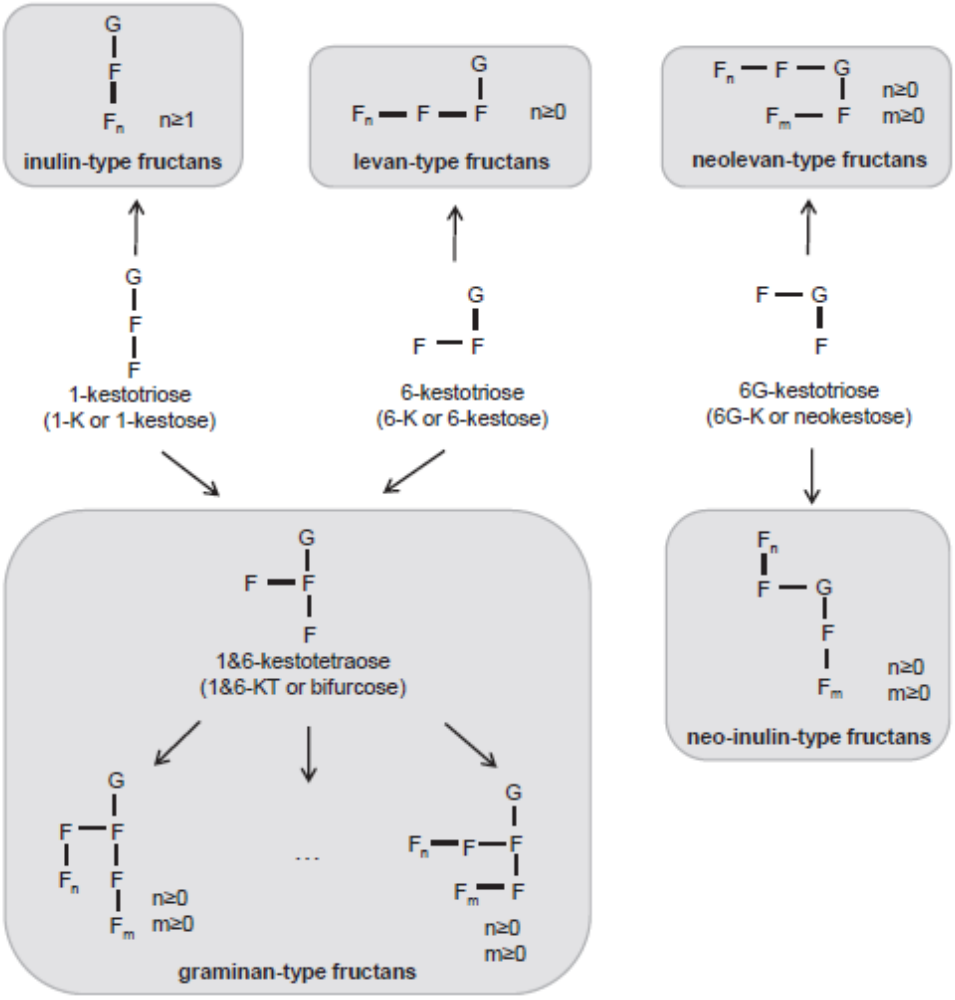


Figure 9 • Overview of the five fructan types. The core of fructan consists of a fructose and sucrose. Depending on the site of attachment of the fructose residue one distinguishes either 1-kestotriose, 6-estotriose, or 6G-kestotriose G: glucose, F: fructose. Vertical and horizontal lines between fructose residues depict β(2,1)-linkages and β(2,6)-linkages respectively. Obtained from Verspreet et al. (2015a)

Wheat is by far the most important fructan source in the Western diet (Stevenson et al. 2012). Most wheat fructans belong to the graminans. In addition, Verspreet et al. (2015) have shown that also both neo-type fructans occur in wheat grains (Verspreet et al. 2015b). Several studies indicate that fructan levels are higher in bran fractions when compared to endosperm, for both wheat and rye (Karppinen et al. 2003, Haska et al. 2008). Within the bran, there are indications for an uneven spread of fructans over the different layers. Two independent studies indicate higher levels of fructan in the aleurone layer compared to pericarp (Glitso & Knudsen 1999, Nordlund et al. 2012).

In the gut, fructans can be degraded by fructanases (inulinases, fructanlyase) which efficiently convert fructan in D-fructose in a single step process (Table 4) (Singh & Gill 2006, Kango & Jain 2011, Flint et al. 2012). Fructanase producing bacteria can be found in the Firmicutes phylum and are mainly gram positive bacteria belonging to the genera, *Streptococcus*, *Bacillus*, *Bifidobacterium*, *Eubacterium*, *Faecalibacterium* and *Clostridium* (Singh & Gill 2006, Moens & De Vuyst 2017), which are all represented in the chicken gut (see below).

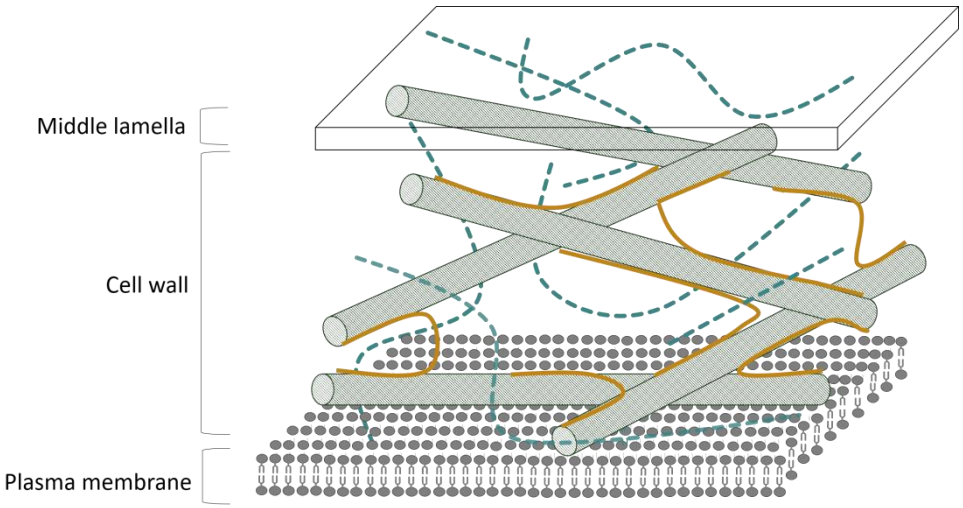


Figure 10 • Plant cell wall structure. Diagrammatic overview of the major structural polysaccharide components of a primary plant cell wall. cellulose, hemicellulose, pectin. Adapted from Flint et al. (2008)

2.2 SELECTIVE COLONIZATION OF WHEAT BRAN

It is well known that microbial communities or biofilms are formed on the surface of insoluble substrates (Kolenbrander et al. 2005, Macfarlane & Macfarlane 2006). Typical for growth of microorganisms in biofilms is that species colonizing the surface of dietary particles are likely to encode a specific enzymatic armory that enables both attachment and degradation of complex insoluble polysaccharides. Degradation products may attract a second wave of bacteria which are specialized in the breakdown of less complex oligosaccharides (Macfarlane & Macfarlane 2006, Flint et al. 2012). The close proximity between the different bacteria fosters an enhanced cross-feeding and more efficient use of substrate and thus more elaborate energy harvest (Macfarlane & Macfarlane 2006).

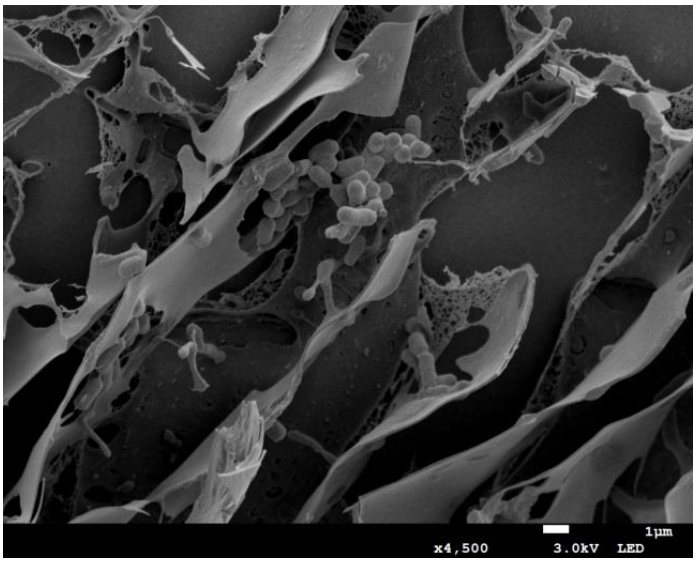


Figure 11 • Scanning electron microscopy image showing rod-shaped bacteria attached to wheat bran. Obtained from De Paepe et al. (unpublished).

Approximately five % of the bacterial cell mass in the lumen of the human large intestine is strongly adhered to the surface of food particles. An even higher percentage is more loosely attached (Macfarlane & Macfarlane 2006). It has been shown previously that this particulate matter harbors an unique and distinct bacterial community (Leitch et al. 2007, Walker et al. 2008, De Paepe et al. 2017). Furthermore, the community composition seems to depend strongly on the nature of the substrate (Leitch et al. 2007, Walker et al. 2008). Leitch et al. (2007) found that wheat bran was specifically colonized by *Clostridium* cluster XIVa species, including uncultured relatives of *Clostridium hathewayi*, *Eubacterium rectale* and *Roseburia* species. Pig gastric mucin and corn starch on the other hand, showed a completely different community profile (Leitch et al. 2007). De Paepe et al. (2017) studied the bacterial community attached to wheat bran and compared it to the planktonic community. In accordance with Leitch et al (2007), they observed an enrichment of *Clostridium* cluster XIVa and depending on the donor, *Prevotella*, *Roseburia*, *Bifidobacterium* and *Bacteroides* species on the bran (De Paepe et al. 2017). Walker et al. (2008) found that members of the *Ruminococcaceae* family were enriched on particulate fecal matter, while the liquid phase was dominated by *Bacteroides* species. In ruminants, it has been shown that specific ruminal bacteria that possess higher fibrolytic activity than the luminal population, develop a dynamic biofilm on digesta particles (Michalet-Doreau et al. 2001, Shinkai & Kobayashi 2007). Michalet-Doreau et al. (2001) observed an enrichment of cellulolytic species in the solid phase of sheep rumen content such as *Fibrobacter succinogenes* and *Ruminococcus flavefaciens*. Macfarlane & Macfarlane (2006) could not find significant differences between the community attached to food residues in human fecal matter and the non-adherent population. Nonetheless, they observed significant differences in fermentation patterns between strongly adherent and non-adherent bacteria: the

fermentation of small, highly soluble oligosaccharides was invariably faster executed by non-adherent bacteria compared to the adhered community (Macfarlane & Macfarlane 2006).

2.3 PROCLAIMED HEALTH EFFECTS OF WHEAT BRAN

Wheat bran is the most concentrated source of DF in the European diet. It is a well-recognized natural healthy food and feed constituent (Stevenson et al. 2012). The European Food Safety Authority (EFSA) has recently approved two health claims related to wheat bran: a causal relationship can be observed between the consumption of wheat bran and

1. the increase in fecal bulk,
2. a reduction in intestinal transit time (EFSA 2010).

Besides the above described mechanical effects, the physiological health effects attributed to wheat bran also relate to its nutritional value (Fardet 2010). Besides fibre, several bioactive compounds can be found in bran. Examples are vitamins (B and E), sulphur containing amino acids (cysteine, methionine), essential amino acids (tryptophan), phenolic acids (ferulic acid) (Table 5). Many of these bran constituents possess anti-oxidative characteristics (Stevenson et al. 2012). The bioavailability of the different bioactive compounds may vary greatly.

Table 5
Average content of major bioactive compounds in wheat bran (%). Adapted from Fardet et al. (2010).

Bioactive compound		Wheat bran
A-Linolenic acid		0.16
Sulfur compounds		0.7
	Total free glutathione	0.038
Dietary fibre		44.6
	Lignins	5.6
	Oligosaccharides	3.7
	Phytic acid	4.2
Minerals and trace elements		3.39
Vitamins		0.0398
	B vitamins	0.0303
	Vitamin E	0.0095
Carotenoids		0.00072
Polyphenols		1.10
	Phenolic acids	1.07
	Flavonoids	0.028
	Lignans	0.0050
Betaine		0.87
Alkylresorcinol		0.27
Total choline		0.17
Total free inositols		0.025
Phytosterols		0.16
Policosanol + metatonin		0.00290
+ para-aminobenzoic acid		
Total		51.5
Subtotal (without DF)		5.9

3. THE INTESTINAL MICROBIOTA OF THE CHICKEN

The intestinal microbiota can be defined as the complex community of microorganisms residing in or passing through the GIT. The gut microbiota is of uttermost importance since it adds metabolic potential to the host (Gerritsen et al. 2011), influences host nutrition and steers gut development and physiology (Kau et al. 2011). The load of bacterial cells present in the GIT is much higher than the number of cells constituting the body of warm-blooded animals. Regarding chickens, the most densely colonized compartments within the gut are the cecal pouches. They contain up to 10^{12} bacteria per gram of cecal contents (Barnes & Impey 1972, Apajalahti et al. 2004). The GIT of chickens is, compared to their body length, shorter than that of most mammals. This implicates a short transit time of less than 3,5h which has important consequences for the digestion process (Clench & Mathias 1992, Clench 1999). Intestinal content is, however, retained longer in the ceca (Clench & Mathias 1995, Clench 1999). Because it is the main site for fermentation and due to the higher bacterial richness and diversity as compared to any other part of the chicken gut, the ceca and its inhabitants have a strong impact on the uptake and utilization of energy and nutrients (Choct et al. 1996, Lan et al. 2005, Shaufi et al. 2015). Hence, during the last decades, the development and composition of the gut microbiota has been extensively studied in chickens focusing mainly on the ceca.

3.1 COMPOSITION OF THE INTESTINAL MICROBIOTA

Chicken gut 16S rRNA gene sequences are deposited in three public databases: Genbank, Silva and Ribosomal Database Project. A total of 915 species-equivalent OTUs belong to 12 phyla and 117 bacterial genera (Wei et al. 2013, Stanley et al. 2014). The three most predominant phyla are Firmicutes, Bacteroidetes and Proteobacteria, accounting, respectively, for 70%, 12.3% and 9.3% of all species (Wei et al. 2013, Choi et al. 2014, Xiao et al. 2017). Genera representing more than 1 % of the Firmicutes are *Clostridium*, *Ruminococcus*, *Lactobacillus*, *Eubacterium*, *Faecalibacerium*, *Butyrivibrio*, *Butyriciccoccus*, *Blautia*, *Hespellia*, *Roseburia* and *Megamonas*. The Proteobacteria phylum is represented mainly by *Desulfohalobium* species while the genera *Bacteroides*, *Prevotella*, *Parabacteroides* and *Alistipes* make up most of the Bacteroidetes phylum. The minor fourth phylum Actinobacteria consists mainly of *Bifidobacterium* species (Wei et al. 2013).

To describe an ecosystem such as the intestinal microbiota, two important terms were introduced: richness and evenness. By employing these two terms one can describe the ecological diversity. At the species level, richness refers to the number of different species present while evenness refers to the abundance of each of the species present (Gerritsen et al. 2011).

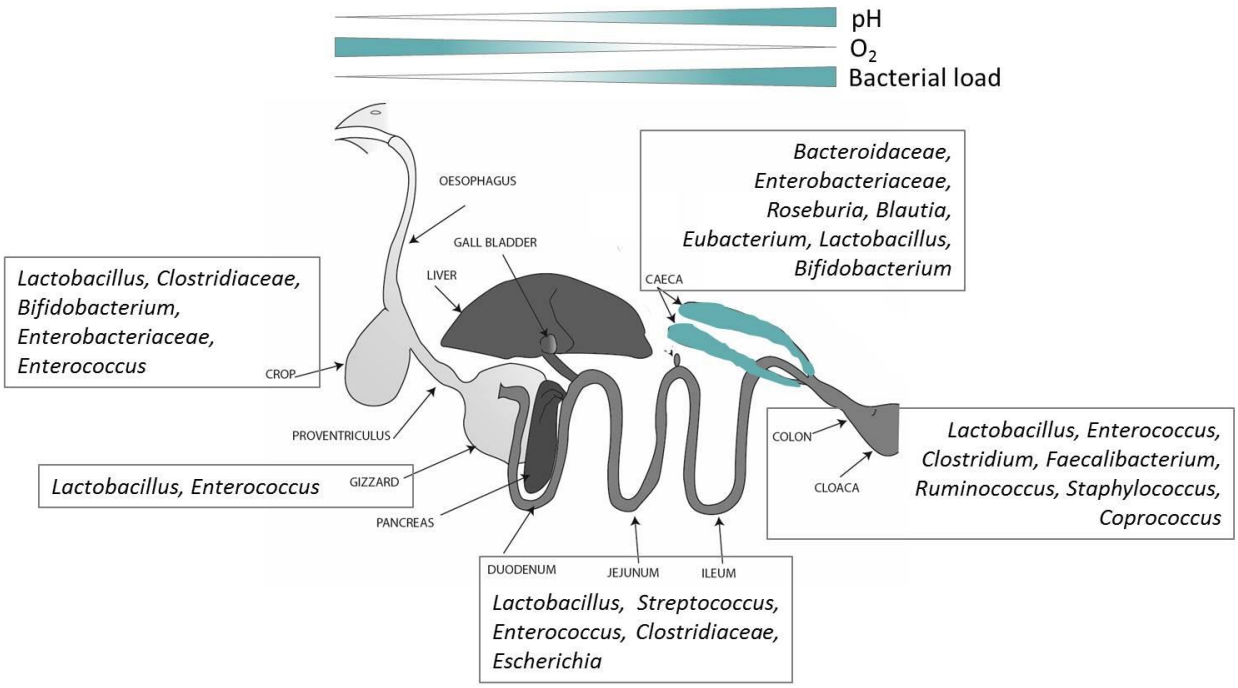


Figure 12 • Schematic representation of the gastrointestinal tract of a chicken. Microbial composition per gut segment is indicated. Adapted from Wei et al. (2013) & Stanley et al. (2014).

Although they are strongly connected to each other, one can consider the different GIT compartments as separate ecosystems (Rehman et al. 2007). The physiology of each compartment determines largely its bacterial composition (Oakley et al. 2014). The **crop**, a transient feed storage organ in which initial starch breakdown occurs, is colonized mainly by *Lactobacillus* species in concentrations of 10^8–10^9 bacteria per gram of content (Figure 12) (van der Wielen et al. 2002, Rehman et al. 2007, Stanley et al. 2014, Svihus 2014). Other species isolated from the crop belong to the genera *Escherichia/Shigella*, *Staphylococcus*, *Klebsiella*, *Enterobacter Bifidobacterium*, *Clostridiaceae* members and *Eubacterium* (Hinton et al. 2000). These are considered to be transient (Hinton et al. 2000). The **proventriculus** and **ventriculus** (gizzard) are the respective glandular and muscular part of the chicken stomach. In the proventriculus feed is mixed with hydrogen chloride and pepsinogen (Rehman et al. 2007, Svihus 2014). In the gizzard the particles of the feed are reduced, although this function is largely lost in broilers

under modern husbandry conditions (Svihus 2014). The pH of the contents varies between 3 and 4 and this low pH poses a barrier to many (pathogenic) bacteria (Rehman et al. 2007). Both ventriculus and proventriculus are colonized mainly by lactobacilli along with enterococci (Figure 12) (Engberg et al. 2000, Rehman et al. 2007). Due to the low pH both bacterial counts and fermentation activity are low when compared to the crop (Rehman et al. 2007). The small intestine can be subdivided in three segments: **duodenum**, **jejunum** and **ileum**. Due to the short transit time and the presence of pancreatic and bile secretions, the bacterial diversity in the duodenum is rather low. Since the influence of these factors diminishes more distally in the intestine, the environment is more favorable for bacterial growth (Svihus 2014). In the ileum, bacterial densities go as high as 10^8-10^9 bacteria per gram content (Apajalahti et al. 2004, Bjerrum et al. 2006). *Lactobacillus* species are predominant in the jejunum and ileum (10^5-10^8 CFU/g). Other facultative anaerobic species belong to the genera *Streptococcus*, *Enterococcus*, and *Escherichia* (van der Wielen et al. 2002, Gong et al. 2007, Rehman et al. 2007, Stanley et al. 2014, Waite & Taylor 2014). Obligate anaerobes belong to the genera *Eubacterium*, *Propionibacterium*, *Clostridium* and *Fusobacterium* (Figure 12) (Rehman et al. 2007, Waite & Taylor 2014). The **ceca** are an important site for water and electrolyte absorption, the recycling of urea and carbohydrate fermentation (Svihus 2014). They are characterized by the highest bacterial density of the entire chicken GIT. Several studies have shown that one gram of cecal contents contains up to 10^{12} colony forming units (CFU) (Barnes & Impey 1972, Apajalahti et al. 2004, Bjerrum et al. 2006, Gong et al. 2007). The constituting bacteria are mainly obligate anaerobes such as members of the phylum Firmicutes, belonging to the genera *Eubacterium*, *Roseburia* and *Blautia*, members of the *Bacteroidaceae* family, but also *Bifidobacterium*, *Lactobacillus* and *Enterobacteriaceae* species (Figure 12) (van der Wielen et al. 2002, Sergeant et al. 2014, Stanley et al. 2014, Waite & Taylor 2014). Herbivorous mammals rely on resident gut microbes to gain energy from their feed and their digestive anatomy and physiology is adjusted accordingly (Flint et al. 2012). In chickens, a similar evolution might have taken place; the development of the specialized cecal pouches provides a niche for extensive microbial fermentation (Stanley et al. 2014). Moreover, due to infrequent emptying of the ceca, only two or three times a day, the retention time in this compartment is generally longer. The result is a high fermentation rate and the production of high amounts of SCFA which are an important energy source for the epithelial cells of the host. The **colon** is, like the ceca, an important site for water absorption. Bacterial species represented in the colon belong to the following genera *Lactobacillus*,

Enterococcus, *Clostridium*, *Faecalibacterium*, *Ruminococcus*, *Staphylococcus* and *Coprococcus*. Lactbacilli and Enterococci are most abundant, constituting 40% and 23% of the total colon microbiome, respectively (Xiao et al. 2017). In fact, the composition of colon contents varies within a day because of cecal emptying and at those time points, it will closely resemble the microbiota composition of the ceca (Stanley et al. 2014, Pauwels et al. 2015).

3.2 DEVELOPMENT OF THE INTESTINAL MICROBIOTA

The colonization of the GIT is thought to start immediately after hatching and so, the environment in which hatching takes place, plays a crucial role in the development of the intestinal microbiota (Apajalahti et al. 2004, Stanley et al. 2013). Among animal production systems, the chicken production system is rather unusual since eggs are separated from the parental flocks prior to hatching. This way, there is a reduced parental influence on the microbiota composition of hatchlings and as a consequence their gut is primarily colonized by environmental bacteria derived from bedding material, feed, human handlers, etc. (Stanley et al. 2014).

Colonization of the GIT occurs very rapidly, already within the first hours post-hatch. One day after hatching, Apajalahti et al. (2004) noted 10^8 and 10^{10} bacteria per gram of content for ileum and cecum respectively. These numbers increased to 10^9 and 10^{12} on day three. During the following 30 days, bacterial counts remain stable but the composition appears to change significantly (Apajalahti et al. 2004). Moreover, bacterial diversity is positively correlated with the age of the chickens (van der Wielen et al. 2002). During the first three days of life, the microbial composition in crop, duodenum and ileum are somewhat similar. The dominating bacterial groups are facultative anaerobic species from the genera *Lactobacillus*, *Streptococcus* and *Enterococcus*, (Rehman et al. 2007, Ranjitkar et al. 2016). In the crop, duodenum and ileum, this remains roughly the same over time except for the fact that numbers of *Streptococcus* and *Enterococcus* species tend to decline. At this point, the ceca are already colonized by a very different community, composed mainly of *Streptococcus* species and *Enterobacteriaceae* and this to a much higher extent when compared to crop, duodenum and ileum (Rehman et al. 2007). From day four on, the composition of the latter three starts to differentiate. This is probably caused by the changing environment within the gut: pH, bile acids, nutrition and oxygen concentration are factors

that can drive the differentiation process (van der Wielen et al. 2002). According to Ranjitkar et al. (2016), seven days post hatch, high numbers of *Lachnospiraceae* (40%), *Ruminococcaceae* (30%), and *Lactobacillaceae* (17%) could be observed. *Streptococcaceae* were also present but in lower abundances (Ranjitkar et al. 2016). Twenty-one days post hatch, 23-55% of the sequences are related to *Faecalibacterium* *(Oakley et al. 2014, Ranjitkar et al. 2016)*. By day 42, equal proportions of *Roseburia* sequences, next to *Eubacterium*, *Lactobacillus*, and *Clostridium* species, mostly obligate anaerobes, could be retrieved (Oakley et al. 2014, Ranjitkar et al. 2016). Numbers of *Enterobacteriaceae* tend to decline over time while species from the phylum Bacteroidetes increase in abundance in the cecal content (Ranjitkar et al. 2016). The presence and abundance of the *Lactobacillaceae* family appears to be constant in the uppermost gut compartments over time (van der Wielen et al. 2002, Oakley et al. 2014, Ranjitkar et al. 2016).

A typical and stable adult gut microbiota is established around three weeks of age (Oakley et al. 2014). The small intestinal microbiota is established faster than the cecal microbiota (around two weeks of age), but eventually the cecal microbial community shows greater diversity in comparison to the ileal community (Lan et al. 2005, Shaufi et al. 2015).

3.3 THE CONTRIBUTION OF THE INTESTINAL MICROBIOTA TOWARDS COLONIZATION RESISTANCE AGAINST PATHOGENS

The intestinal microbiota is inextricably linked to health and disease. A “healthy” microbiota protects individuals from colonization and infection by enteric pathogens, and this mechanism is often referred to as colonization resistance (Lawley & Walker 2013). Both non-immune mediated and immune mediated mechanisms play a role in maintaining a healthy gut and all dominant bacterial phyla of the GIT are involved (Buffie & Pamer 2013).

3.3.1 NON-IMMUNE MEDIATED MECHANISMS

Metabolic exclusion by production of bacteriocins and SCFA. The microbiota can confer colonization resistance by the production and secretion of antibacterial molecules such as bacteriocins and their smaller counterparts, microcins. Numerous reports have supported the antimicrobial activity of (purified) bacteriocins both *in vitro* and *in vivo* (Schamberger & Diez-Gonzalez 2004, Cursino et al. 2006, Millette et al. 2008, Pickard et al. 2017). In general these molecules have narrow activity spectra, affecting closely related bacteria (Schamberger & Diez-Gonzalez 2004). For

example, the microcin-producing *Escherichia coli* strain Nissle 1917 could limit growth of competitors such as commensal *E. coli*, adherent-invasive *E. coli* and the related pathogen *Salmonella enterica* in an inflamed intestine (Sassone-Corsi et al. 2016).

Bacterial metabolic byproducts can also have inhibitory effects. In sufficiently high concentrations, SCFA are known to inhibit the growth of, among others, (enterohaemorhaggic) *E. coli*, *Samonella* and *Clostridium difficile* (Cherrington et al. 1991, Shin et al. 2002). Bacteria constantly sense signals from their surroundings and adjust gene expression accordingly. Intrinsic metabolic activities of the commensal microbiome can create conditions which affect virulence expression of pathogens as well. It has been shown, for example, that both propionate and butyrate can suppress *Salmonella* pathogenicity island 1 gene expression, a gene cluster involved in epithelial cell invasion (Gantois et al. 2006, Hung et al. 2013).

Competition for nutrients and niches. Besides protecting themselves against detrimental molecules, bacterial pathogens must compete for the same nutrient sources with the commensal community in order to colonize the GIT. Commensals are well adapted to host physical and nutritional constraints and therefore, under normal circumstances, can outcompete invading pathogens. Ng et al. (2013) have demonstrated that sialic acid and fucose, liberated from host glycans by symbionts like *Bacteroides* species, were an important sugar source for *Salmonella* Typhimurium and *Clostridium difficile*. These mucosal carbohydrates became only available to the pathogens when the bacteria that normally consume them were depleted by an antibiotic treatment (Ng et al. 2013). This mechanism clearly demonstrates the competition for nutrients.

Both commensals and pathogens share the same ecological niche and compete for binding sites on gastrointestinal mucins. Cell surface mucins are transmembrane glycoproteins with an extensively O-glycosylated extracellular domain, which serves as ligand for bacterial adhesins, and a cytoplasmic domain for signal transduction. Glycosylation patterns are shown to be influenced by the microbiota composition (Arike et al. 2017, Ubeda et al. 2017). By occupying these niches, the healthy microbiota can impede attachment of pathogenic bacteria to the intestinal epithelium and subsequent invasion. Extracellular appendages such as flagella, fimbriae or pili play a major role in the attachment of bacteria to their host and

have been implicated in mucus adhesion (Juge 2012). Heterologous expression of *Bacillus cereus* CH flagellin in *Lactococcus lactis* for example, has been shown to result in increased adherence to mucin-coated plates and increased the ability to competitively inhibit the adhesion of pathogenic *E. coli* and *Salmonella enterica* (Sanchez et al. 2011). Not only is pathogen binding hampered by direct competition for mucus adhesion sites, mannan derived products are widely accepted to prevent *Salmonella* colonization by binding to the adhesin FimH present on the fimbriae (Juge 2012, Badia et al. 2013). For example, it has been shown that *Saccharomyces boulardii*-adhesion to *Salmonella* triggered fecal elimination along with decreased bacterial translocation (Pontier-Bres et al. 2014). In other words, scavenging of the pathogen resulted in reduced intestinal attachment and invasion.

3.3.2 IMMUNE MEDIATED MECHANISMS

Vaishnava et al. (2008) suggested that commensals protect their host against pathogen invasion not only through colonization resistance but also by activating MyD88-dependent signaling in the Paneth cells, triggering expression of a complex antimicrobial program that includes $RegIII_{\gamma}$ and $RegIII_{\beta}$ lectins (Vaishnava et al. 2008). In a recent study, Wang and others provided evidence for the presence of Paneth cells in the chicken small intestine (Wang et al. 2016). The antimicrobial molecules appear to have limited effect on the luminal microbiota, since they are captured in the mucus barrier, facilitating high local peptide concentration on vulnerable mucosal surfaces, while still allowing the presence of an enteric microbiota (Meyer-Hoffert et al. 2008). Key host receptors in this process are Toll-like receptors (TLRs) and nucleotide-binding oligomerization domain-like (NOD) receptors, which are strategically placed on the epithelial interface and sense intestinal microbes and their products (e.g. LPS). How commensals are discriminated from pathogens is often poorly understood.

The commensal microbiota can affect adaptive immunity as well. Most evidence was gathered from work with germ free mice. For example, it has been shown that the intestinal microbiota can steer the differentiation of T cells into different subsets, e.g. Th17 cells or Tregs (Honda & Littman 2016). Th17 cells are pro-inflammatory, secrete IL-17 and IL-22 and increase colonization resistance, while Tregs temper inflammation to avoid excessive tissue damage by the secretion of IL-10 and TGF-β (Honda & Littman 2016, Ubeda et al. 2017). The delicate balance between pro- and anti-inflammatory mechanisms, crucial for gut immune homeostasis, is affected by the composition of the commensal microbial community (Arpaia et

al. 2013). A population of *Clostridium*-related bacteria, segmented filamentous bacteria (SFB) are known to stimulate Th17 differentiation (Ivanov et al. 2009), while Treg development is driven by Clostridial species from cluster IV, XIVa and XVIII (Atarashi et al. 2011, Atarashi et al. 2013). The mechanism for the latter process most probably relies on the production of acetate, propionate and butyrate, which have been shown to enhance TGF-β expression by intestinal epithelial cells, inducing in its turn Treg differentiation (Atarashi et al. 2013). Another mechanism relies on the histon deacetylase inhibitory activity of butyrate, which can increase histone H3 acetylation in the promotor of Foxp3, a master regulator of Treg development (Arpaia et al. 2013, Furusawa et al. 2013). SCFA have also been implicated in the development of B cells and antibody production. They have been shown to steer B cell differentiation into IgA producing plasma cells (Kim et al. 2016). Production of IgA by B cells is key for the host to control infections on mucosal surfaces, including the gastrointestinal tract (Cerutti & Rescigno 2008).

3.4 ROLE OF THE MICROBIOTA IN DIETARY FIBRE DIGESTION

3.4.1 CARBOHYDRATE ACTIVE ENZYMES (CAZYMES)

Chickens lack most of the enzymatic machinery to degrade structural polysaccharides derived from plant material. Therefore, these components end up undigested in the hindgut where they are fermented by the resident microbiota. Bacterial degradation of non-starch polysaccharides is orchestrated by an innate set of carbohydrate-active enzymes (CAZymes) (Table 4) (Lombard et al. 2014). Both the abundance and fitness of intestinal bacteria strongly depend on the ability to produce specific CAZymes (Bjursell et al. 2006) because bacterial taxa capable of producing a wide range of enzymes will easily outcompete other organisms when dietary polysaccharides are scarce (Sonnenburg & Sonnenburg 2014). The CAZy database distinguishes three main classes of CAZymes: (1) Glycoside hydrolases (GHs), (2) polysaccharide lyases (PLs) and (3) carbohydrate esterases (CE) (Lombard et al. 2014). GHs cleave glycosidic bonds by the insertion of a water molecule, while PLs cleave complex carbohydrates using a β-elimination mechanism (Henrissat 1991, Lombard et al. 2010, Koropatkin et al. 2012). CEs remove ester substituents from the glycan chain to facilitate the actions of GHs and PLs (Lairson et al. 2008). The breakdown of complex carbohydrates, such as AX, requires the collaboration of several enzymes often produced by different bacterial species (Flint et al. 2008, Martens et al. 2011). The expression of these genes is tightly regulated depending not only on substrate availability but also in response to the host and the presence of other bacteria in

the gut (Flint et al. 2012). This means that the same set of genes may carry out different functions depending on the context of the community (Fischbach & Sonnenburg 2011). According to the CAZy database, the genome of *Gallus gallus* encodes GHs belonging to 17 different families (Lombard et al. 2014) whereas *Bacteroides thetaiotaomicron*, a bacterial species with notorious carbohydrate degradation activities, encodes GHs belonging to as many as 56 different families (Xu et al. 2003). As a matter of fact, many members of the Bacteroidetes phylum are equipped with an extended armory of CAZymes (Xu et al. 2003, Flint et al. 2008, Flint et al. 2012, El Kaoutari et al. 2013). The carbohydrate catabolism of Firmicutes may not be underestimated either. *Roseburia intestinalis* for example, is reported for extensive xylan-utilization (Duncan et al. 2002). Furthermore, several studies indicate that specific members within the Firmicutes tend to occur associated with AX-rich material such as wheat bran (Leitch et al. 2007, Walker et al. 2008). (see also chapter 2.2). This indicates that they encode the appropriate carbohydrate binding modules and/or hydrolases to start degradation. In addition to Bacteroidetes and Firmicutes species, Kaoutari et al. (2013) have shown that *Bifidobacterium* species, such as *B. longum*, are also very much involved in carbohydrate metabolism by encoding several putative GHs and PLs (El Kaoutari et al. 2013). Nevertheless, very little is known about which bacterial genera and species are actually involved in the initial and secondary attack of carbohydrate polymers from specific plant sources.

3.4.2 TROPHIC CHAINS WITHIN THE GUT

Plant derived carbohydrates stimulate not only the so-called primary degrading bacteria, but also other metabolically less versatile taxa can be enhanced through cross-feeding (Fischbach & Sonnenburg 2011). This cross-feeding covers the transfer of fermentation products such as

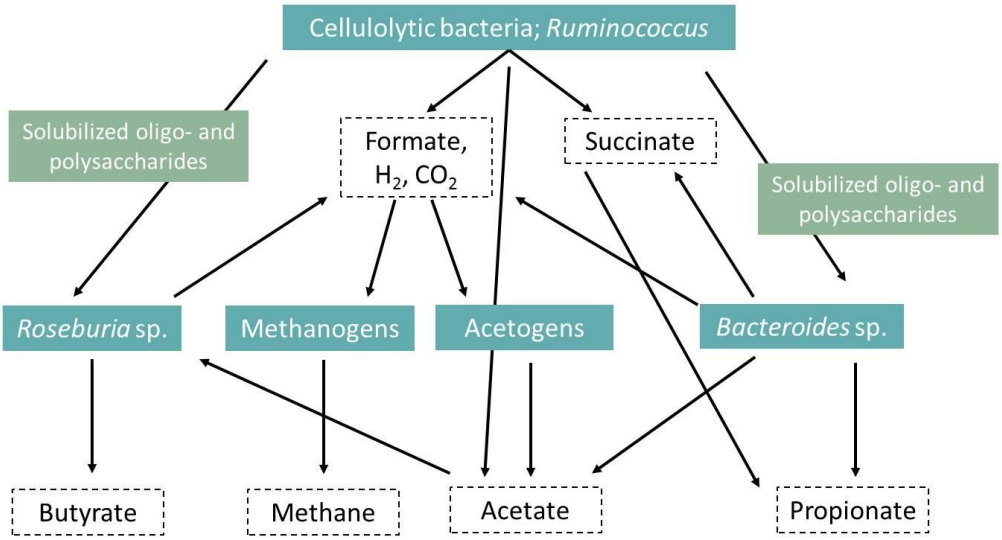


Figure 13 • Simplified scheme illustrating the relationships between primary degraders of insoluble plant fibre and other members of gut microbial communities. Adapted from Flint et al. (2008).

hydrogen, lactate and partially degraded substrates to other members of the intestinal community (Belenguer et al. 2006, Falony et al. 2006). As an example, cellulolytic species often are closely associated with the plant surface (Cheng et al. 1984). To access the cellulose fibrils within the complex plant material, these bacteria need to degrade the surrounding matrix polysaccharides (xylan, pectins…). The resulting solubilized products are mostly not consumed by the cellulolytic bacteria but become available for other members of the community (Osborne & Dehority 1989, Belenguer et al. 2006, Falony et al. 2006). Among the Firmicutes, cellulolytic *Ruminnococcus* species have been noted to be considerably enriched on fibre rich fractions (Larue et al. 2005). Species like *Ruminococcus flavefaciens* have the ability to degrade cellulose and a range of plant cell wall polysaccharides, by encoding a cellulosome system (Rincon et al. 2003). This system harbors both xylanolytic and cellulolytic enzymes, tightly bound to a scaffolding protein (Chapter 2.1.3) (Beguin & Aubert 1994).

Once monosaccharides, such as glucose, are imported into the bacterial cell, they will be targeted to either one of three glycolytic pathways: the Embden-Meyerhof-Parnas pathway (EMP), the pentose phosphate pathway (PP) or the Entner-Doudoroff pathway (ED). All three

pathways generate phosphoenolpyruvate (PEP), which is a central precursor for the production of alcohols and organic acids, the main products of saccharolytic fermentation (Figure 13) (Fischbach & Sonnenburg 2011, Wolfe 2015). A fourth pathway, the fructose-6-phosphate shunt, has a limited taxonomic distribution in the colonic microbiota and is restricted to members of the genus *Bifidobacterium*. It enables the conversion of PEP to lactate and acetate (Macfarlane & Macfarlane 2003). Bacteria residing in the gut are adapted to a low partial oxygen pressure. In contrast to facultative anaerobes, strict anaerobes often lack the ability to establish electrochemical gradients to obtain ATP. Instead they apply substrate level phosphorylation to reduce metabolic intermediates to regenerate NAD^+ (Macfarlane & Macfarlane 2003). Substrate level phosphorylation is relatively inefficient in terms of energy conservation. This implies that large amounts of substrate need to be converted for growth, resulting in high levels of metabolic end products (Macfarlane & Macfarlane 2003, Fischbach & Sonnenburg 2011). These end products are mainly the SCFA: acetate, propionate and butyrate (Macfarlane & Macfarlane 2003, Duncan et al. 2007). Lactate, ethanol and succinate are intermediate fermentation products that rarely will accumulate since they are readily metabolized to SCFA by cross-feeding organisms (Topping & Clifton 2001). Lactate, for example, can be converted to butyrate by a specific group of bacteria within the *Lachnospiraceae* family (Duncan et al. 2004, De Maesschalck et al. 2015a). Another important intermediate product is hydrogen. The accumulation of hydrogen inhibits the metabolism of the primary degraders. Thus, a clearance and redistribution is of uttermost importance. Methanogenic Archaea, acetogenic bacteria and sulfate reducing bacteria compete for hydrogen from the environment and convert it to methane, acetate and hydrogen sulfide, respectively (Dar et al. 2008). There will be different physiological implications depending on whichever contestant wins this competition (Dore et al. 1995). Acetate is beneficial in terms of energy harvest, while it has been described that hydrogen sulfide can have detrimental effects on gut health.

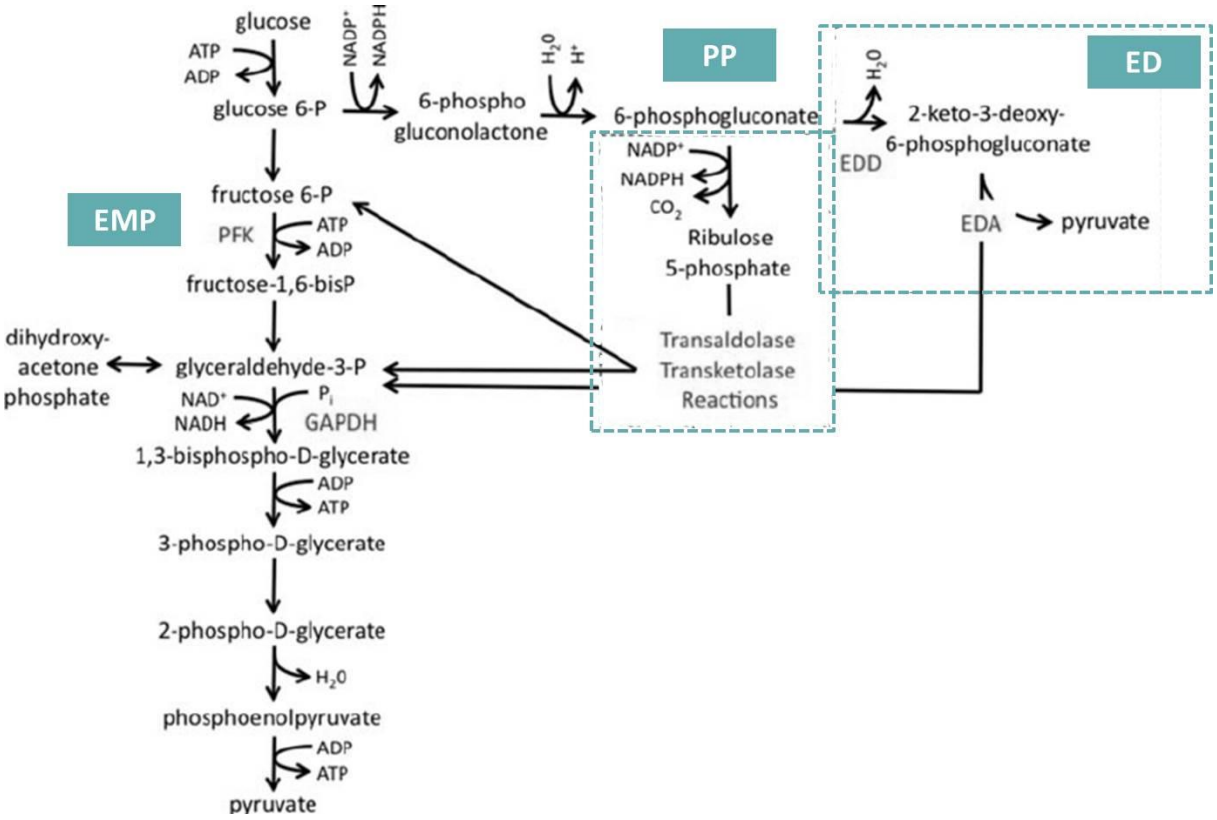


Figure 14 • Bacterial glycolytic pathways. In the EMP pathway glucose is converted to pyruvate with the net synthesis of two ATP molecules. In the ED pathway glucose-6-phosphate is oxidized to generate pyruvate and glyceraldehyde-3-phosphate, yielding only one ATP molecule. In the PP pathway 6—phosphogluconate is broken down to ribulose-5-phosphate and provides ribose. The boxes highlight reactions unique to the PP and ED pathways. EMP: Embden-Meyerhof-Parnas, ED: Entner-Doudoroff pathway, PP: pentose phosphate pathway. PEP, phosphoenolpyruvate; PFK, phosphofructokinase; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; EDD, 6-phosphogluconate dehydratase; EDA, 2-keto 3-deoxy-D-gluconate 6-phosphate aldolase. Adapted from Wolfe (2015).

In conclusion, carbohydrates accessible to the microbiota act as selective agents that can alter the microbiota composition. In addition they can also dictate the functionality and metabolic outcome in the gut (Sonnenburg & Sonnenburg 2014). These observations have led to the current application of specific plant cell-wall polysaccharides as prebiotic dietary additives to steer the gut microbiota in a direction that is deemed to be beneficial to health (Kruse et al. 1999).

REFERENCES

Abt, M.C. and Artis, D. The Dynamic Influence of Commensal Bacteria on the Immune Response to Pathogens. (2013) *Current opinion in microbiology* **16**, 1: 4-9.

Akhtar, M., Tariq, A.F., Awais, M.M., Iqbal, Z., Muhammad, F., Shahid, M. and Hiszczynska-Sawicka, E. Studies on Wheat Bran Arabinoxylan for Its Immunostimulatory and Protective Effects against Avian Coccidiosis. (2012) *Carbohydrate Polymers* **90**, 1: 333-9.

Amerah, A.M., Ravindran, V. and Lentle, R.G. Influence of Insoluble Fibre and Whole Wheat Inclusion on the Performance, Digestive Tract Development and Ileal Microbiota Profile of Broiler Chickens. (2009) *British Poultry Science* **50**, 3: 366-375.

American Association of Cereal Chemists. The Definition of Dietary Fiber. (2001) *Cereal Food World* **46**: 112-26.

Ao, Z. and Choct, M. Oligosaccharides Affect Performance and Gut Development of Broiler Chickens. (2013) *Asian-Australasian Journal of Animal Sciences* **26**, 1: 116-121.

Apajalahti, J., Kettunen, A. and Graham, H. Characteristics of the Gastrointestinal Microbial Communities, with Special Reference to the Chicken. (2004) *Worlds Poultry Science Journal* **60**, 2: 223-232.

Arike, L., Holmen-Larsson, J. and Hansson, G.C. Intestinal Muc2 Mucin O-Glycosylation Is Affected by Microbiota and Regulated by Differential Expression of Glycosyltranferases. (2017) *Glycobiology* **27**, 4: 318-328.

Arpaia, N., Campbell, C., Fan, X., Dikiy, S., van der Veeken, J., deRoos, P., Liu, H., Cross, J.R., Pfeffer, K., Coffer, P.J. and Rudensky, A.Y. Metabolites Produced by Commensal Bacteria Promote Peripheral Regulatory T-Cell Generation. (2013) *Nature* **504**, 7480: 451-5.

Ata, S., Din, M.I., Rasool, A., Qasim, I. and Ul Mohsin, I. Equilibrium, Thermodynamics, and Kinetic Sorption Studies for the Removal of Coomassie Brilliant Blue on Wheat Bran as a Low-Cost Adsorbent. (2012) *Journal of Analytical Methods in Chemistry*.

Atarashi, K., Tanoue, T., Oshima, K., Suda, W., Nagano, Y., Nishikawa, H., Fukuda, S., Saito, T., Narushima, S., Hase, K., Kim, S., Fritz, J.V., Wilmes, P., Ueha, S., Matsushima, K., Ohno, H., Olle, B., Sakaguchi, S., Taniguchi, T., Morita, H., Hattori, M. and Honda, K. Treg Induction by a Rationally Selected Mixture of Clostridia Strains from the Human Microbiota. (2013) *Nature* **500**, 7461: 232-6.

Atarashi, K., Tanoue, T., Shima, T., Imaoka, A., Kuwahara, T., Momose, Y., Cheng, G., Yamasaki, S., Saito, T., Ohba, Y., Taniguchi, T., Takeda, K., Hori, S., Ivanov, II, Umesaki, Y., Itoh, K. and Honda, K. Induction of Colonic Regulatory T Cells by Indigenous Clostridium Species. (2011) *Science* **331**, 6015: 337-41.

Badia, R., Lizardo, R., Martínez, P. and Brufau, J. Oligosaccharide Structure Determines Prebiotic Role of B-Galactomannan against Salmonella Enterica Ser. Typhimurium in Vitro. (2013) *Gut Microbes* **4**, 1: 72-75.

Bailey, J.S., Blankenship, L.C. and Cox, N.A. Effect of Fructooligosaccharide on *Salmonella* Colonization of the Chicken Intestine. (1991) *Poultry Science* **70**, 12: 2433-2438.

Bajaj, V., Lucas, R.L., Hwang, C. and Lee, C.A. Co-Ordinate Regulation of *Salmonella* Typhimurium Invasion Genes by Environmental and Regulatory Factors Is Mediated by Control of *Hila* Expression. (1996) *Molecular Microbiology* **22**, 4: 703-14.

Barnes, E.M. and Impey, C.S. Some Properties of the Nonsporing Anaerobes from Poultry Caeca. (1972) *Journal of Applied Microbiology* **35**, 2: 241-51.

Beckmann, L., Simon, O. and Vahjen, W. Isolation and Identification of Mixed Linked Beta -Glucan Degrading Bacteria in the Intestine of Broiler Chickens and Partial Characterization of Respective 1,3-1,4-Beta -Glucanase Activities. (2006) *Journal of Basic Microbiology* **46**, 3: 175-85.

Beguin, P. and Aubert, J.P. The Biological Degradation of Cellulose. (1994) *Fems Microbiology Reviews* **13**, 1: 25-58.

Belenguer, A., Duncan, S.H., Calder, A.G., Holtrop, G., Louis, P., Lobley, G.E. and Flint, H.J. Two Routes of Metabolic Cross-Feeding between *Bifidobacterium Adolescentis* and Butyrate-Producing Anaerobes from the Human Gut. (2006) *Applied and Environmental Microbiology* **72**, 5: 3593-3599.

Biely, P., Mislovicova, D. and Toman, R. Soluble Chromogenic Substrates for the Assay of Endo-1,4-Beta-Xylanases and Endo-1,4-Beta-Glucanases. (1985) *Analytical Biochemistry* **144**, 1: 142-146.

Biggs, P., Parsons, C.M. and Fahey, G.C. The Effects of Several Oligosaccharides on Growth Performance, Nutrient Digestibilities, and Cecal Microbial Populations in Young Chicks. (2007) *Poultry Science* **86**, 11: 2327-2336.

Bindels, L.B., Delzenne, N.M., Cani, P.D. and Walter, J. Towards a More Comprehensive Concept for Prebiotics. (2015) *Nature Reviews Gastroenterology & Hepatology* **12**, 5: 303-310.

Bjerrum, L., Engberg, R.M., Leser, T.D., Jensen, B.B., Finster, K. and Pedersen, K. Microbial Community Composition of the Ileum and Cecum of Broiler Chickens as Revealed by Molecular and Culture-Based Techniques. (2006) *Poultry Science* **85**, 7: 1151-1164.

Bjerrum, L., Pedersen, K. and Engberg, R.M. The Influence of Whole Wheat Feeding on *Salmonella* Infection and Gut Flora Composition in Broilers. (2005) *Avian Diseases* **49**, 1: 9-15.

Bjursell, M.K., Martens, E.C. and Gordon, J.I. Functional Genomic and Metabolic Studies of the Adaptations of a Prominent Adult Human Gut Symbiont, *Bacteroides Thetaiotaomicron*, to the Suckling Period. (2006) *Journal of Biological Chemistry* **281**, 47: 36269-36279.

Buffie, C.G. and Pamer, E.G. Microbiota-Mediated Colonization Resistance against Intestinal Pathogens. (2013) *Nature Reviews Immunology* **13**, 11: 790-801.

Burkinshaw, B.J. and Strynadka, N.C.J. Assembly and Structure of the T3ss. (2014) *Biochimica Et Biophysica Acta-Molecular Cell Research* **1843**, 8: 1649-1663.

Burrin, D.G., Petersen, Y., Stoll, B. and Sangild, P. Glucagon-Like Peptide 2: A Nutrient-Responsive Gut Growth Factor. (2001) *Journal of Nutrition* **131**, 3: 709-712.

Caprez, A., Arrigoni, E., Amado, R. and Neukom, H. Influence of Different Types of Thermal-Treatment on the Chemical-Composition and Physical-Properties of Wheat Bran. (1986) *Journal of Cereal Science* **4**, 3: 233-239.

Caprita, A. and Caprita, R. The Effect of Thermal Processing on Soluble Dietary Fibre Fraction in Wheat. (2011) *Journal of Food Agriculture & Environment* **9**, 3-4: 14-15.

Cerutti, A. and Rescigno, M. The Biology of Intestinal Immunoglobulin a Responses. (2008) *Immunity* **28**, 6: 740-50.

Chambers, J.R., Spencer, J.L. and Modler, H.W. The Influence of Complex Carbohydrates on *Salmonella* Typhimurium Colonization, Ph, and Density of Broiler Ceca. (1997) *Poultry Science* **76**, 3: 445-451.

Cheng, K.J., Stewart, C.S., Dinsdale, D. and Costerton, J.W. Electron Microscopy of Bacteria Involved in the Digestion of Plant Cell Walls. (1984) *Animal Feed Science and Technology* **10**, 2: 93-120.

Cherrington, C.A., Hinton, M., Pearson, G.R. and Chopra, I. Short-Chain Organic Acids at Ph 5.0 Kill Escherichia Coli and Salmonella Spp. Without Causing Membrane Perturbation. (1991) *Journal of Applied Bacteriology* **70**, 2: 161-5.

Cho, J.H., Zhang, Z.F. and Kim, I.H. Effects of Single or Combined Dietary Supplementation of -Glucan and Kefir on Growth Performance, Blood Characteristics and Meat Quality in Broilers. (2013) *British Poultry Science* **54**, 2: 216-221.

Choct, M., Hughes, R.J., Wang, J., Bedford, M.R., Morgan, A.J. and Annison, G. Increased Small Intestinal Fermentation Is Partly Responsible for the Anti-Nutritive Activity of Non-Starch Polysaccharides in Chickens. (1996) *British Poultry Science* **37**, 3: 609-21.

Choi, J.H., Kim, G.B. and Cha, C.J. Spatial Heterogeneity and Stability of Bacterial Community in the Gastrointestinal Tracts of Broiler Chickens. (2014) *Poultry Science* **93**, 8: 1942-1950.

Ciolacu, D., Gorgieva, S., Tampu, D. and Kokol, V. Enzymatic Hydrolysis of Different Allomorphic Forms of Microcrystalline Cellulose. (2011) *Cellulose* **18**, 6: 1527-1541.

Cleemput, G., Roels, S.P., Vanoort, M., Grobet, P.J. and Delcour, J.A. Heterogeneity in the Structure of Water-Soluble Arabinoxylans in European Wheat Flours of Variable Bread-Making Quality. (1993) *Cereal Chemistry* **70**, 3: 324-329.

Clench, M.H. The Avian Cecum: Update and Motility Review. (1999) *Journal of Experimental Zoology* **283**, 4-5: 441-447.

Clench, M.H. and Mathias, J.R. Intestinal Transit - How Can It Be Delayed Long Enough for Birds to Act as Long-Distance Disperal Agents. (1992) *The Auk* **109**, 4: 933-936.

Clench, M.H. and Mathias, J.R. The Avian Cecum - a Review. (1995) *Wilson Bulletin* **107**, 1: 93-121.

Codex Alimentarius Commission. "Report of the 31th Session of the Codex Committee on Nutrition and Foods for Special Dietary Uses." In *Codex Alimentarius Commission*. Rome, **2009**.

Colleoni-Sirghie, M., Fulton, D.B. and White, P.J. Structural Features of Water Soluble (1,3) (1,4)-Beta-D-Glucans from High-Beta-Glucan and Traditional Oat Lines. (2003) *Carbohydrate Polymers* **54**, 2: 237-249.

Cotta, M.A. Utilization of Xylooligosaccharides by Selected Ruminal Bacteria. (1993) *Applied and Environmental Microbiology* **59**, 11: 3557-3563.

Cotta, M.A. and Zeltwanger, R.L. Degradation and Utilization of Xylan by the Ruminal Bacteria Butyrivibrio-Fibrisolvens and Selenomonas-Ruminantium. (1995) *Applied and Environmental Microbiology* **61**, 12: 4396-4402.

Courtin, C.M., Broekaert, W.F., Swennen, K., Lescroart, O., Onagbesan, O., Buyse, J., Decuypere, E., Van de Wiele, T., Marzorati, M., Verstraete, W., Huyghebaert, G. and Delcour, J.A. Dietary Inclusion of Wheat Bran Arabinoxylooligosaccharides Induces Beneficial Nutritional Effects in Chickens. (2008a) *Cereal Chemistry* **85**, 5: 607-613.

Courtin, C.M., Swennen, K., Broekaert, W.F., Swennen, Q., Buyse, J., Decuypere, E., Michiels, C.W., Ketelaere, B.D. and Delcour, J.A. Effects of Dietary Inclusion of Xylooligosaccharides, Arabinoxylooligoscaccharides and Soluble Arabinoxylan on the Microbial Composition of Caecal Contents of Chickens. (2008b) *Journal of the Science of Food and Agriculture* **88**, 14: 2517-2522.

Crittenden, R., Karppinen, S., Ojanen, S., Tenkanen, M., Fagerstrom, R., Matto, J., Saarela, M., Mattila-Sandholm, T. and Poutanen, K. In Vitro Fermentation of Cereal Dietary Fibre Carbohydrates by Probiotic and Intestinal Bacteria. (2002) *Journal of the Science of Food and Agriculture* **82**, 8: 781-789.

Cursino, L., Smajs, D., Smarda, J., Nardi, R.M., Nicoli, J.R., Chartone-Souza, E. and Nascimento, A.M. Exoproducts of the Escherichia Coli Strain H22 Inhibiting Some Enteric Pathogens Both in Vitro and in Vivo. (2006) *J Appl Microbiol* **100**, 4: 821-9.

Damen, B., Verspreet, J., Pollet, A., Broekaert, W.F., Delcour, J.A. and Courtin, C.M. Prebiotic Effects and Intestinal Fermentation of Cereal Arabinoxylans and Arabinoxylan Oligosaccharides in Rats Depend Strongly on Their Structural Properties and Joint Presence. (2011) *Molecular Nutrition & Food Research* **55**, 12: 1862-1874.

Dar, S.A., Kleerebezem, R., Stams, A.J., Kuenen, J.G. and Muyzer, G. Competition and Coexistence of Sulfate-Reducing Bacteria, Acetogens and Methanogens in a Lab-Scale Anaerobic Bioreactor as Affected by Changing Substrate to Sulfate Ratio. (2008) *Appl Microbiol Biotechnol* **78**, 6: 1045-55.

Darwin, K.H. and Miller, V.L. Molecular Basis of the Interaction of Salmonella with the Intestinal Mucosa. (1999) *Clinical Microbiology Reviews* **12**, 3: 405-+.

De Maesschalck, C., Eeckhaut, V., Maertens, L., De Lange, L., Marchal, L., Nezer, C., De Baere, S., Croubels, S., Daube, G., Dewulf, J., Haesebrouck, F., Ducatelle, R., Taminau, B. and Van Immerseel, F. Effects of Xylo-Oligosaccharides on Broiler Chicken Performance and Microbiota. (2015a) *Applied Environmental Microbiology* **81**, 17: 5880-5888.

De Maesschalck, C., Eeckhaut, V., Maertens, L., De Lange, L., Marchal, L., Nezer, C., De Baere, S., Croubels, S., Daube, G., Dewulf, J., Haesebrouck, F., Ducatelle, R., Taminau, B. and Van Immerseel, F. Effects of Xylo-Oligosaccharides on Broiler Chicken Performance and Microbiota. (2015b) *Applied and Environmental Microbiology* **81**, 17: 5880-5888.

De Paepe, K., Kerckhof, F.M., Verspreet, J., Courtin, C.M. and Van de Wiele, T. Inter-Individual Differences Determine the Outcome of Wheat Bran Colonization by the Human Gut Microbiome. (2017) *Environmental Microbiology*.

De Preter, V., Hamer, H.M., Windey, K. and Verbeke, K. The Impact of Pre- and/or Probiotics on Human Colonic Metabolism: Does It Affect Human Health? (2011) *Molecular Nutrition & Food Research* **55**, 1: 46-57.

Derrien, M. and Vlieg, J. Fate, Activity, and Impact of Ingested Bacteria within the Human Gut Microbiota. (2015) *Trends in Microbiology* **23**, 6: 354-366.

DeVries, J.W., Prosky, L., Li, B. and Cho, S. A Historical Perspective on Defining Dietary Fiber. (1999) *Cereal Foods World* **44**, 5: 367-369.

Dikeman, C.L. and Fahey, G.C. Viscosity as Related to Dietary Fiber: A Review. (2006) *Crit Rev Food Sci Nutr* **46**, 8: 649-63.

Dore, J., Pochart, P., Bernalier, A., Goderel, I., Morvan, B. and Rambaud, J.C. Enumeration of H-2-Utilizing Methanogenic Archaea, Acetogenic and Sulfate-Reducing Bacteria from Human Feces. (1995) *Fems Microbiology Ecology* **17**, 4: 279-284.

Dornez, E., Gebruers, K., Delcour, J.A. and Courtin, C.A. Grain-Associated Xylanases: Occurrence, Variability, and Implications for Cereal Processing. (2009) *Trends in Food Science & Technology* **20**, 11-12: 495-510.

Drucker, D.J., Ehrlich, P., Asa, S.L. and Brubaker, P.L. Induction of Intestinal Epithelial Proliferation by Glucagon-Like Peptide 2. (1996) *Proceedings of the National Academy of Sciences of the United States of America* **93**, 15: 7911-7916.

Duncan, S.H., Hold, G.L., Barcenilla, A., Stewart, C.S. and Flint, H.J. *Roseburia Intestinalis* Sp Nov., a Novel Saccharolytic, Butyrate-Producing Bacterium from Human Faeces. (2002) *International Journal of Systematic and Evolutionary Microbiology* **52**: 1615-1620.

Duncan, S.H., Louis, P. and Flint, H.J. Lactate-Utilizing Bacteria, Isolated from Human Feces, That Produce Butyrate as a Major Fermentation Product. (2004) *Applied and Environmental Microbiology* **70**, 10: 5810-5817.

Duncan, S.H., Louis, P. and Flint, H.J. Cultivable Bacterial Diversity from the Human Colon. (2007) *Letters in Applied Microbiology* **44**, 4: 343-350.

Eeckhaut, V., Van Immerseel, F., Dewulf, J., Pasmans, F., Haesebrouck, F., Ducatelle, R., Courtin, C.M., Delcour, J.A. and Broekaert, W.F. Arabinoxylooligosaccharides from Wheat Bran Inhibit *Salmonella* Colonization in Broiler Chickens. (2008) *Poultry Science* **87**, 11: 2329-34.

EFSA. Scientific Opinion on the Substantiation of Health Claims Related to Wheat Bran Fibre and Increase in Faecal Bulk (Id 3066), Reduction in Intestinal Transit Time (Id 828, 839, 3067, 4699) and Contribution to the Maintenance or Achievement of a Normal Body Weight (Id 829) Pursuant to Article 13(1) of Regulation (Ec) No 1924/2006. (2010) *EFSA Journal* **8**, 10: 1817-n/a.

EFSA. The 2013 Joint Ecdc/Efsa Report on Trends and Sources of Zoonoses, Zoonotic Agents and Food-Borne Outbreaks Published. (2015) *EuroSurveillance* **20**, 4.

El Kaoutari, A., Armougom, F., Gordon, J.I., Raoult, D. and Henrissat, B. The Abundance and Variety of Carbohydrate-Active Enzymes in the Human Gut Microbiota. (2013) *Nature Reviews Microbiology* **11**, 7: 497-504.

Elleuch, M., Bedigian, D., Roiseux, O., Besbes, S., Blecker, C. and Attia, H. Dietary Fibre and Fibre-Rich by-Products of Food Processing: Characterisation, Technological Functionality and Commercial Applications: A Review. (2011) *Food Chemistry* **124**, 2: 411-421.

Engberg, R.M., Hedemann, M.S., Leser, T.D. and Jensen, B.B. Effect of Zinc Bacitracin and Salinomycin on Intestinal Microflora and Performance of Broilers. (2000) *Poultry Science* **79**, 9: 1311-1319.

Englyst, H.N., Trowell, H., Southgate, D.A.T. and Cummings, J.H. Dietary Fiber and Resistant Starch. (1987) *American Journal of Clinical Nutrition* **46**, 6: 873-874.

Enslin, O. Über Einen Apparatzur Messung Der Flüssigkeitsaufnahme Von Quellbaren Und Porösen Stoffen Und Zur Charakterisierung Der Benetzbarkeit. (1933) *Die Chemische Fabrik* **13**: 147-148.

Erlandson, K.A., Delamarre, S.C. and Batt, C.A. Genetic Evidence for a Defective Xylan Degradation Pathway in Lactococcus Lactis. (2001) *Applied and Environmental Microbiology* **67**, 4: 1445-1452.

EU. Commission Directive 2008/100/Ecof 28 October 2008 Amending Council Directive 90/496/Eec on Nutrition Labelling for Foodstuffs as Regards Recommended Daily Allowances, Energy Conversion Factors and Definitions. (2008) *Official Journal of the European Union* **285**: 9-12.

Fabrega, A. and Vila, J. *Salmonella Enterica* Serovar Typhimurium Skills to Succeed in the Host: Virulence and Regulation. (2013) *Clinical Microbiology Reviews* **26**, 2: 308-341.

Fahlen, T.F., Mathur, N. and Jones, B.D. Identification and Characterization of Mutants with Increased Expression of *Hila*, the Invasion Gene Transcriptional Activator of *Salmonella* Typhimurium. (2000) *FEMS Immunology & Medical Microbiology* **28**, 1: 25-35.

Falony, G., Vlachou, A., Verbrugghe, K. and De Vuyst, L. Cross-Feeding between *Bifidobacterium Longum* Bb536 and Acetate-Converting, Butyrate-Producing Colon Bacteria During Growth on Oligofructose. (2006) *Applied and Environmental Microbiology* **72**, 12: 7835-7841.

Farajzadeh, M.A. and Monji, A.B. Adsorption Characteristics of Wheat Bran Towards Heavy Metal Cations. (2004) *Separation and Purification Technology* **38**, 3: 197-207.

Fardet, A. New Hypotheses for the Health-Protective Mechanisms of Whole-Grain Cereals: What Is Beyond Fibre? (2010) *Nutrition Research Reviews* **23**, 1: 65-134.

Feng, Z.Q., Dou, W., Alaxi, S., Niu, Y.G. and Yu, L.L. Modified Soluble Dietary Fiber from Black Bean Coats with Its Rheological and Bile Acid Binding Properties. (2017) *Food Hydrocolloids* **62**: 94-101.

Fernandez, F., Hinton, M. and Van Gils, B. Dietary Mannan-Oligosaccharides and Their Effect on Chicken Caecal Microflora in Relation to Salmonella Enteritidis Colonization. (2002) *Avian Pathology* **31**, 1: 49-58.

Fischbach, M.A. and Sonnenburg, J.L. Eating for Two: How Metabolism Establishes Lnterspecies Interactions in the Gut. (2011) *Cell Host & Microbe* **10**, 4: 336-347.

Flint, H.J., Bayer, E.A., Rincon, M.T., Lamed, R. and White, B.A. Polysaccharide Utilization by Gut Bacteria: Potential for New Insights from Genomic Analysis. (2008) *Nature Reviews Microbiology* **6**, 2: 121-131.

Flint, H.J., Scott, K.P., Duncan, S.H., Louis, P. and Forano, E. Microbial Degradation of Complex Carbohydrates in the Gut. (2012) *Gut Microbes* **3**, 4: 289-306.

Foley, S.L., Lynne, A.M. and Nayak, R. Salmonella Challenges: Prevalence in Swine and Poultry and Potential Pathogenicity of Such Isolates. (2008) *J Anim Sci* **86**, 14 Suppl: E149-62.

Foster, J.W. Salmonella Acid Shock Proteins Are Required for the Adaptive Acid Tolerance Response. (1991) *Journal of Bacteriology* **173**, 21: 6896-6902.

Furusawa, Y., Obata, Y., Fukuda, S., Endo, T.A., Nakato, G., Takahashi, D., Nakanishi, Y., Uetake, C., Kato, K., Kato, T., Takahashi, M., Fukuda, N.N., Murakami, S., Miyauchi, E., Hino, S., Atarashi, K., Onawa, S., Fujimura, Y., Lockett, T., Clarke, J.M., Topping, D.L., Tomita, M., Hori, S., Ohara, O., Morita, T., Koseki, H., Kikuchi, J., Honda, K., Hase, K. and Ohno, H. Commensal Microbe-Derived Butyrate Induces the Differentiation of Colonic Regulatory T Cells. (2013) *Nature* **504**, 7480: 446-50.

Galan, J.E. and Curtiss, R., 3rd. Expression of *Salmonella* Typhimurium Genes Required for Invasion Is Regulated by Changes in DNA Supercoiling. (1990) *Infection and Immunity* **58**, 6: 1879-85.

Gantois, I., Ducatelle, R., Pasmans, F., Haesebrouck, F., Hautefort, I., Thompson, A., Hinton, J.C. and Van Immerseel, F. Butyrate Specifically Down-Regulates *Salmonella* Pathogenicity Island 1 Gene Expression. (2006) *Applied and Environmental Microbiology* **72**, 1: 946-9.

Geier, M.S., Torok, V.A., Allison, G.E., Ophel-Keller, K. and Hughes, R.J. Indigestible Carbohydrates Alter the Intestinal Microbiota but Do Not Influence the Performance of Broiler Chickens. (2009) *Journal of Applied Microbiology* **106**, 5: 1540-1548.

Gerritsen, J., Smidt, H., Rijkers, G.T. and de Vos, W.M. Intestinal Microbiota in Human Health and Disease: The Impact of Probiotics. (2011) *Genes Nutr* **6**, 3: 209-40.

Gibson, G.R., Probert, H.M., Loo, J.V., Rastall, R.A. and Roberfroid, M.B. Dietary Modulation of the Human Colonic Microbiota: Updating the Concept of Prebiotics. (2004) *Nutrition Research Reviews* **17**, 2: 259-75.

Glitso, L.V. and Knudsen, K.E.B. Milling of Whole Grain Rye to Obtain Fractions with Different Dietary Fibre Characteristics. (1999) *Journal of Cereal Science* **29**, 1: 89-97.

Gong, J.H., Si, W.D., Forster, R.J., Huang, R.L., Yu, H., Yin, Y.L., Yang, C.B. and Han, Y.M. 16s Rrna Gene-Based Analysis of Mucosa-Associated Bacterial Community and Phylogeny in the Chicken Gastrointestinal Tracts: From Crops to Ceca. (2007) *Fems Microbiology Ecology* **59**, 1: 147-157.

Gonzalez-Alvarado, J.M., Jimenez-Moreno, E., Lazaro, R. and Mateos, G.G. Effect of Type of Cereal, Heat Processing of the Cereal, and Inclusion of Fiber in the Diet on Productive Performance and Digestive Traits of Broilers. (2007) *Poultry Science* **86**, 8: 1705-1715.

Gonzalez-Alvarado, J.M., Jimenez-Moreno, E., Royon, F.D., Lazaro, R. and Mateos, G.G. Effects of Dietary Oat Hulls and Sugar Beet Pulp on Productive Performance and Nutrient Digestibility of Broilers from 1 to 42 D of Age. (2010) *Journal of Dairy Science* **93**: 495-495.

Greiner, R. and Konietzny, U. Phytase for Food Application. (2006) *Food Technology and Biotechnology* **44**, 2: 125-140.

Grootaert, C., Van den Abbeele, P., Marzorati, M., Broekaert, W.F., Courtin, C.M., Delcour, J.A., Verstraete, W. and Van de Wiele, T. Comparison of Prebiotic Effects of Arabinoxylan Oligosaccharides and Inulin in a Simulator of the Human Intestinal Microbial Ecosystem. (2009) *Fems Microbiology Ecology* **69**, 2: 231-242.

Guillon, F. and Champ, M. Structural and Physical Properties of Dietary Fibres, and Consequences of Processing on Human Physiology. (2000) *Food Research International* **33**, 3-4: 233-245.

Guilloteau, P., Martin, L., Eeckhaut, V., Ducatelle, R., Zabielski, R. and Van Immerseel, F. From the Gut to the Peripheral Tissues: The Multiple Effects of Butyrate. (2010) *Nutr Res Rev* **23**, 2: 366-84.

Hamaker, B.R. and Tuncil, Y.E. A Perspective on the Complexity of Dietary Fiber Structures and Their Potential Effect on the Gut Microbiota. (2014) *Journal of Molecular Biology* **426**, 23: 3838-3850.

Hamer, H.M., Jonkers, D., Troost, F., Bast, A., Vanhoutvin, S., Venema, K. and Brummer, R.J. Butyrate Modulates Oxidative Stress in the Colonic Mucosa of Healthy Humans. (2008) *European Journal of Gastroenterology & Hepatology* **20**, 7: A11-A11.

Haska, L., Nyman, M. and Andersson, R. Distribution and Characterisation of Fructan in Wheat Milling Fractions. (2008) *Journal of Cereal Science* **48**, 3: 768-774.

Havrlentova, M., Petrulakova, Z., Burgarova, A., Gago, F., Hlinkova, A. and Sturdik, E. Cereal Beta-Glucans and Their Significance for the Preparation of Functional Foods - a Review. (2011) *Czech Journal of Food Sciences* **29**, 1: 1-14.

Hayashi, H., Abe, T., Sakamoto, M., Ohara, H., Ikernura, T., Sakka, K. and Benno, Y. Direct Cloning of Genes Encoding Novel Xylanases from the Human Gut. (2005) *Canadian Journal of Microbiology* **51**, 3: 251-259.

Hemdane, S., Jacobs, P.J., Dornez, E., Verspreet, J., Delcour, J.A. and Courtin, C.M. Wheat (Triticum Aestivum L.) Bran in Bread Making: A Critical Review. (2016) *Comprehensive Reviews in Food Science and Food Safety* **15**, 1: 28-42.

Henrissat, B. A Classification of Glycosyl Hydrolases Based on Amino-Acid-Sequence Similarities. (1991) *Biochemical Journal* **280**: 309-316.

Henrissat, B. and Bairoch, A. Updating the Sequence-Based Classification of Glycosyl Hydrolases. (1996) *Biochemical Journal* **316**: 695-696.

Hetland, H., Svihus, B. and Choct, M. Role of Insoluble Fiber on Gizzard Activity in Layers. (2005) *Journal of Applied Poultry Research* **14**, 1: 38-46.

Hetland, H., Svihus, B. and Krogdahl, A. Effects of Oat Hulls and Wood Shavings on Digestion in Broilers and Layers Fed Diets Based on Whole or Ground Wheat. (2003) *Br Poult Sci* **44**, 2: 275-82.

Himmel, M.E. Biomass Recalcitrance: Engineering Plants and Enzymes for Biofuels Production (Vol 315, Pg 804, 2007). (2007) *Science* **316**, 5827: 982-982.

Hinton, A., Jr., Buhr, R.J. and Ingram, K.D. Physical, Chemical, and Microbiological Changes in the Crop of Broiler Chickens Subjected to Incremental Feed Withdrawal. (2000) *Poult Sci* **79**, 2: 212-8.

Hipsley, E.H. Dietary "Fibre" and Pregnancy Toxaemia. (1953) *Br Med J* **2**, 4833: 420-2.

Honda, K. and Littman, D.R. The Microbiota in Adaptive Immune Homeostasis and Disease. (2016) *Nature* **535**, 7610: 75-84.

Huang, D.S., Li, D.F., Xing, J.J., Ma, Y.X., Li, Z.J. and Lv, S.Q. Effects of Feed Particle Size and Feed Form on Survival of *Salmonella* Typhimurium in the Alimentary Tract and Cecal *S*. Typhimurium Reduction in Growing Broilers. (2006) *Poultry Science* **85**, 5: 831-836.

Hughes, S.A., Shewry, P.R., Li, L., Gibson, G.R., Sanz, M.L. and Rastall, R.A. *In Vitro* Fermentation by Human Fecal Microflora of Wheat Arabinoxylans. (2007) *Journal of Agricultural and Food Chemistry* **55**, 11: 4589-4595.

Hung, C.C., Garner, C.D., Slauch, J.M., Dwyer, Z.W., Lawhon, S.D., Frye, J.G., McClelland, M., Ahmer, B.M. and Altier, C. The Intestinal Fatty Acid Propionate Inhibits Salmonella Invasion through the Post-Translational Control of Hild. (2013) *Molecular Microbiology* **87**, 5: 1045-60.

Inan, M.S., Rasoulpour, R.J., Yin, L., Hubbard, A.K., Rosenberg, D.W. and Giardina, C. The Luminal Short-Chain Fatty Acid Butyrate Modulates Nf-Kappa B Activity in a Human Colonic Epithelial Cell Line. (2000) *Gastroenterology* **118**, 4: 724-734.

Ivanov, II, Atarashi, K., Manel, N., Brodie, E.L., Shima, T., Karaoz, U., Wei, D., Goldfarb, K.C., Santee, C.A., Lynch, S.V., Tanoue, T., Imaoka, A., Itoh, K., Takeda, K., Umesaki, Y., Honda, K. and Littman, D.R. Induction of Intestinal Th17 Cells by Segmented Filamentous Bacteria. (2009) *Cell* **139**, 3: 485-98.

Izydorczyk, M.S. and Dexter, J.E. Barley Beta-Glucans and Arabinoxylans: Molecular Structure, Physicochemical Properties, and Uses in Food Products-a Review. (2008) *Food Research International* **41**, 9: 850-868.

Jacobs, P.J., Bogaerts, S., Hemdane, S., Delcour, J.A. and Courtin, C.M. Impact of Wheat Bran Hydration Properties as Affected by Toasting and Degree of Milling on Optimal Dough Development in Bread Making. (2016a) *Journal of Agricultural and Food Chemistry* **64**, 18: 3636-3644.

Jacobs, P.J., Hemdane, S., Delcour, J.A. and Courtin, C.M. Dry Heat Treatment Affects Wheat Bran Surface Properties and Hydration Kinetics. (2016b) *Food Chemistry* **203**: 513-520.

Jacobs, P.J., Hemdane, S., Dornez, E., Delcour, J.A. and Courtin, C.M. Study of Hydration Properties of Wheat Bran as a Function of Particle Size. (2015) *Food Chemistry* **179**: 296-304.

Jiang, Z.Y., Applegate, T.J. and Lossie, A.C. Cloning, Annotation and Developmental Expression of the Chicken Intestinal *Muc2* Gene. (2013) *Plos One* **8**, 1.

Jimenez-Moreno, E., Gonzalez-Alvarado, J.M., Gonzalez-Sanchez, D., Lazaro, R. and Mateos, G.G. Effects of Type and Particle Size of Dietary Fiber on Growth Performance and Digestive Traits of Broilers from 1 to 21 Days of Age. (2010) *Poultry Science* **89**, 10: 2197-212.

Jimenez-Moreno, E., Gonzalez-Alvarado, J.M., Lazaro, R. and Mateos, G.G. Effects of Type of Cereal, Heat Processing of the Cereal, and Fiber Inclusion in the Diet on Gizzard Ph and Nutrient Utilization in Broilers at Different Ages. (2009) *Poultry Science* **88**, 9: 1925-1933.

Johansson, L., Virkki, L., Maunu, S., Lehto, M., Ekholm, P. and Varo, P. Structural Characterization of Water Soluble Beta-Glucan of Oat Bran. (2000) *Carbohydrate Polymers* **42**, 2: 143-148.

Jones, J.M. Codex-Aligned Dietary Fiber Definitions Help to Bridge the 'Fiber Gap'. (2014) *Nutr J* **13**: 34.

Juge, N. Microbial Adhesins to Gastrointestinal Mucus. (2012) *Trends in Microbiology* **20**, 1: 30-39.

Kamada, N., Chen, G.Y., Inohara, N. and Nunez, G. Control of Pathogens and Pathobionts by the Gut Microbiota. (2013) *Nature Immunology* **14**, 7: 685-690.

Kango, N. and Jain, S.C. Production and Properties of Microbial Inulinases: Recent Advances. (2011) *Food Biotechnology* **25**, 3: 165-212.

Kaplan, H. and Hutkins, R.W. Fermentation of Fructooligosaccharides by Lactic Acid Bacteria and Bifidobacteria. (2000) *Applied and Environmental Microbiology* **66**, 6: 2682-2684.

Karppinen, S., Myllymaki, O., Forssell, P. and Poutanen, K. Fructan Content of Rye and Rye Products. (2003) *Cereal Chemistry* **80**, 2: 168-171.

Kau, A.L., Ahern, P.P., Griffin, N.W., Goodman, A.L. and Gordon, J.I. Human Nutrition, the Gut Microbiome, and Immune System: Envisioning the Future. (2011) *Nature* **474**, 7351: 327-336.

Kim, G.B., Seo, Y.M., Kim, C.H. and Paik, I.K. Effect of Dietary Prebiotic Supplementation on the Performance, Intestinal Microflora, and Immune Response of Broilers. (2011) *Poultry Science* **90**, 1: 75-82.

Kim, M., Qie, Y., Park, J. and Kim, C.H. Gut Microbial Metabolites Fuel Host Antibody Responses. (2016) *Cell Host Microbe* **20**, 2: 202-14.

Klampfer, L., Huang, J., Sasazuki, T., Shirasawa, S. and Augenlicht, L. Inhibition of Interferon Gamma Signaling by the Short Chain Fatty Acid Butyrate. (2003) *Molecular Cancer Research* **1**, 11: 855-862.

Klasing, K.C. Nutritional Modulation of Resistance to Infectious Diseases. (1998) *Poultry Science* **77**, 8: 1119-1125.

Kleessen, B., Hartmann, L. and Blaut, M. Oligofructose and Long-Chain Inulin: Influence on the Gut Microbial Ecology of Rats Associated with a Human Faecal Flora. (2001) *British Journal of Nutrition* **86**, 2: 291-300.

Knudsen, K.E.B. Fiber and Nonstarch Polysaccharide Content and Variation in Common Crops Used in Broiler Diets. (2014) *Poultry Science* **93**, 9: 2380-2393.

Kobayashi, Y., Okuda, N., Matsumoto, M., Inoue, K., Wakita, M. and Hoshino, S. Constitutive Expression of a Heterologous *Eubacterium Ruminantium* Xylanase Gene (Xyna) in *Butyrivibrio Fibrisolvens*. (1998) *Fems Microbiology Letters* **163**, 1: 11-17.

Kolenbrander, P.E., Egland, P.G., Diaz, P.I. and Palmer, R.J. Genome-Genome Interactions: Bacterial Communities in Initial Dental Plaque. (2005) *Trends in Microbiology* **13**, 1: 11-15.

Koropatkin, N.M., Cameron, E.A. and Martens, E.C. How Glycan Metabolism Shapes the Human Gut Microbiota. (2012) *Nature Reviews Microbiology* **10**, 5: 323-335.

Kruse, H.P., Kleessen, B. and Blaut, M. Effects of Inulin on Faecal Bifidobacteria in Human Subjects. (1999) *British Journal of Nutrition* **82**, 5: 375-382.

Kumar, R., Singh, S. and Singh, O.V. Bioconversion of Lignocellulosic Biomass: Biochemical and Molecular Perspectives. (2008) *Journal of Industrial Microbiology & Biotechnology* **35**, 5: 377-391.

Lairson, L.L., Henrissat, B., Davies, G.J. and Withers, S.G. "Glycosyltransferases: Structures, Functions, and Mechanisms." In *Annual Review of Biochemistry*, 77, 521-555. Palo Alto: Annual Reviews, 2008.

Lan, Y., Verstegen, M.W.A., Tamminga, S. and Williams, B.A. The Role of the Commensal Gut Microbial Community in Broiler Chickens. (2005) *Worlds Poultry Science Journal* **61**, 1: 95-104.

LaRock, D.L., Chaudhary, A. and Miller, S.I. Salmonellae Interactions with Host Processes. (2015) *Nature Reviews Microbiology* **13**, 4: 191-205.

Larue, R., Yu, Z., Parisi, V.A., Egan, A.R. and Morrison, M. Novel Microbial Diversity Adherent to Plant Biomass in the Herbivore Gastrointestinal Tract, as Revealed by Ribosomal Intergenic Spacer Analysis and Rrs Gene Sequencing. (2005) *Environmental Microbiology* **7**, 4: 530-43.

Lattimer, J.M. and Haub, M.D. Effects of Dietary Fiber and Its Components on Metabolic Health. (2010) *Nutrients* **2**, 12: 1266-1289.

Lawhon, S.D., Maurer, R., Suyemoto, M. and Altier, C. Intestinal Short-Chain Fatty Acids Alter *Salmonella* Typhimurium Invasion Gene Expression and Virulence through *Bara*/*Sira*. (2002) *Molecular Microbiology* **46**, 5: 1451-1464.

Lawley, T.D. and Walker, A.W. Intestinal Colonization Resistance. (2013) *Immunology* **138**, 1: 1-11.

Lazaridou, A. and Biliaderis, C.G. Molecular Aspects of Cereal Beta-Glucan Functionality: Physical Properties, Technological Applications and Physiological Effects. (2007) *Journal of Cereal Science* **46**, 2: 101-118.

Leitch, E.C.M., Walker, A.W., Duncan, S.H., Holtrop, G. and Flint, H.J. Selective Colonization of Insoluble Substrates by Human Faecal Bacteria. (2007) *Environmental Microbiology* **9**, 3: 667-679.

Lewis, D.H. Nomenclature and Diagrammatic Representation of Oligomeric Fructans - a Paper for Discussion. (1993) *New Phytologist* **124**, 4: 583-594.

Liljebjelke, K.A., Hofacre, C.L., Liu, T.R., White, D.G., Ayers, S., Young, S. and Maurer, J.J. Vertical and Horizontal Transmission of *Salmonella* within Integrated Broiler Production System. (2005) *Foodborne Pathogens and Disease* **2**, 1: 90-102.

Liu, H., Fu, S.Y., Zhu, J.Y., Li, H. and Zhan, H.Y. Visualization of Enzymatic Hydrolysis of Cellulose Using Afm Phase Imaging. (2009) *Enzyme and Microbial Technology* **45**, 4: 274-281.

Lombard, V., Bernard, T., Rancurel, C., Brumer, H., Coutinho, P.M. and Henrissat, B. A Hierarchical Classification of Polysaccharide Lyases for Glycogenomics. (2010) *Biochemical Journal* **432**: 437-444.

Lombard, V., Ramulu, H.G., Drula, E., Coutinho, P.M. and Henrissat, B. The Carbohydrate-Active Enzymes Database (Cazy) in 2013. (2014) *Nucleic Acids Research* **42**, D1: D490-D495.

Lostroh, C.P. and Lee, C.A. The *Salmonella* Pathogenicity Island-1 Type Iii Secretion System. (2001) *Microbes and Infection* **3**, 14-15: 1281-1291.

Macfarlane, S. and Macfarlane, G.T. Regulation of Short-Chain Fatty Acid Production. (2003) *Proceedings of the Nutrition Society* **62**, 1: 67-72.

Macfarlane, S. and Macfarlane, G.T. Composition and Metabolic Activities of Bacterial Biofilms Colonizing Food Residues in the Human Gut. (2006) *Applied and Environmental Microbiology* **72**, 9: 6204-6211.

Maes, C. and Delcour, J.A. Alkaline Hydrogen Peroxide Extraction of Wheat Bran Non-Starch Polysaccharides. (2001) *Journal of Cereal Science* **34**, 1: 29-35.

Maes, C. and Delcour, J.A. Structural Characterisation of Water-Extractable and Water-Unextractable Arabinoxylans in Wheat Bran. (2002) *Journal of Cereal Science* **35**, 3: 315-326.

Marin, C., Balasch, S., Vega, S. and Lainez, M. Sources of *Salmonella* Contamination During Broiler Production in Eastern Spain. (2011) *Preventive Veterinary Medicine* **98**, 1: 39-45.

Martens, E.C., Lowe, E.C., Chiang, H., Pudlo, N.A., Wu, M., McNulty, N.P., Abbott, D.W., Henrissat, B., Gilbert, H.J., Bolam, D.N. and Gordon, J.I. Recognition and Degradation of Plant Cell Wall Polysaccharides by Two Human Gut Symbionts. (2011) *PLoS Biol* **9**, 12: e1001221.

Mateos, G.G., Jimenez-Moreno, E., Serrano, M.P. and Lazaro, R.P. Poultry Response to High Levels of Dietary Fiber Sources Varying in Physical and Chemical Characteristics. (2012) *Journal of Applied Poultry Research* **21**, 1: 156-174.

McCleary, B.V. The Evolution of Dietary Fiber Definitions and Methods and the Role of Aacc International. (2011) *Cereal Foods World* **56**, 3: 103-103.

McCleary, B.V., DeVries, J.W., Rader, J.I., Cohen, G., Prosky, L., Mugford, D.C., Champ, M. and Okuma, K. Determination of Total Dietary Fiber (Codex Definition) by Enzymatic-Gravimetric Method and Liquid Chromatography: Collaborative Study. (2010) *Journal of Aoac International* **93**, 1: 221-233.

Medie, F.M., Davies, G.J., Drancourt, M. and Henrissat, B. Genome Analyses Highlight the Different Biological Roles of Cellulases. (2012) *Nature Reviews Microbiology* **10**, 3: 227-U.

Meerburg, B.G. and Kijlstra, A. Role of Rodents in Transmission of *Salmonella* and *Campylobacter*. (2007) *Journal of the Science of Food and Agriculture* **87**, 15: 2774-2781.

Meyer-Hoffert, U., Hornef, M.W., Henriques-Normark, B., Axelsson, L.G., Midtvedt, T., Putsep, K. and Andersson, M. Secreted Enteric Antimicrobial Activity Localises to the Mucus Surface Layer. (2008) *Gut* **57**, 6: 764-71.

Michalet-Doreau, B., Fernandez, I., Peyron, C., Millet, L. and Fonty, G. Fibrolytic Activities and Cellulolytic Bacterial Community Structure in the Solid and Liquid Phases of Rumen Contents. (2001) *Reproduction, Nutrition, Development* **41**, 2: 187-94.

Millette, M., Cornut, G., Dupont, C., Shareck, F., Archambault, D. and Lacroix, M. Capacity of Human Nisin- and Pediocin-Producing Lactic Acid Bacteria to Reduce Intestinal Colonization by Vancomycin-Resistant Enterococci. (2008) *Appl Environ Microbiol* **74**, 7: 1997-2003.

Miyazaki, K., Martin, J.C., Marinsek-Logar, R. and Flint, H.J. Degradation and Utilization of Xylans by the Rumen Anaerobe *Prevotella Bryantii* (Formerly *P. Ruminicola* Subsp *Brevis*) B(1)4. (1997) *Anaerobe* **3**, 6: 373-381.

Moens, F. and De Vuyst, L. Inulin-Type Fructan Degradation Capacity of *Clostridium* Cluster Iv and Xiva Butyrate-Producing Colon Bacteria and Their Associated Metabolic Outcomes. (2017) *Benef Microbes* **8**, 3: 473-490.

Munroe, D.G., Gupta, A.K., Kooshesh, F., Vyas, T.B., Rizkalla, G., Wang, H., Demchyshyn, L., Yang, Z.J., Kamboj, R.K., Chen, H.Y., McCallum, K., Sumner-Smith, M., Drucker, D.J. and Crivici, A. Prototypic G Protein-Coupled Receptor for the Intestinotrophic Factor Glucagon-Like Peptide 2. (1999) *Proceedings of the National Academy of Sciences of the United States of America* **96**, 4: 1569-1573.

Ng, K.M., Ferreyra, J.A., Higginbottom, S.K., Lynch, J.B., Kashyap, P.C., Gopinath, S., Naidu, N., Choudhury, B., Weimer, B.C., Monack, D.M. and Sonnenburg, J.L. Microbiota-Liberated Host Sugars Facilitate Post-Antibiotic Expansion of Enteric Pathogens. (2013) *Nature* **502**, 7469: 96-+.

Nordlund, E., Aura, A.M., Mattila, I., Kosso, T., Rouau, X. and Poutanen, K. Formation of Phenolic Microbial Metabolites and Short-Chain Fatty Acids from Rye, Wheat, and Oat Bran and Their Fractions in the Metabolical in Vitro Colon Model. (2012) *Journal of Agricultural and Food Chemistry* **60**, 33: 8134-8145.

Oakley, B.B., Buhr, R.J., Ritz, C.W., Kiepper, B.H., Berrang, M.E., Seal, B.S. and Cox, N.A. Successional Changes in the Chicken Cecal Microbiome During 42 Days of Growth Are Independent of Organic Acid Feed Additives. (2014) *BMC Vet Res* **10**: 282.

Osborne, J.M. and Dehority, B.A. Synergism in Degradation and Utilization of Intact Forage Cellulose, Hemicellulose, and Pectin by Three Pure Cultures of Ruminal Bacteria. (1989) *Applied and Environmental Microbiology* **55**, 9: 2247-2250.

Osullivan, A.C. Cellulose: The Structure Slowly Unravels. (1997) *Cellulose* **4**, 3: 173-207.

Panato, A., Antonini, E., Bortolotti, F. and Ninfali, P. The Histology of Grain Caryopses for Nutrient Location: A Comparative Study of Six Cereals. (2017) *International Journal of Food Science and Technology* **52**, 5: 1238-1245.

Pauwels, J., Taminiau, B., Janssens, G.P.J., De Beenhouwer, M., Delhalle, L., Daube, G. and Coopman, F. Cecal Drop Reflects the Chickens' Cecal Microbiome, Fecal Drop Does Not. (2015) *Journal of Microbiological Methods* **117**: 164-170.

Peng, L., He, Z., Chen, W., Holzman, I.R. and Lin, J. Effects of Butyrate on Intestinal Barrier Function in a Caco-2 Cell Monolayer Model of Intestinal Barrier. (2007) *Pediatr Res* **61**, 1: 37-41.

Pickard, J.M., Zeng, M.Y., Caruso, R. and Nunez, G. Gut Microbiota: Role in Pathogen Colonization, Immune Responses, and Inflammatory Disease. (2017) *Immunol Rev* **279**, 1: 70-89.

Pinkert, A., Marsh, K.N., Pang, S.S. and Staiger, M.P. Ionic Liquids and Their Interaction with Cellulose. (2009) *Chemical Reviews* **109**, 12: 6712-6728.

Pollet, A., Van Craeyveld, V., Van de Wiele, T., Verstraete, W., Delcour, J.A. and Courtin, C.M. In Vitro Fermentation of Arabinoxylan Oligosaccharides and Low Molecular Mass Arabinoxylans with Different Structural Properties from Wheat (Triticum Aestivum L.) Bran and Psyllium (Plantago Ovata Forsk) Seed Husk. (2012) *J Agric Food Chem* **60**, 4: 946-54.

Pontier-Bres, R., Munro, P., Boyer, L., Anty, R., Imbert, V., Terciolo, C., André, F., Rampal, P., Lemichez, E., Peyron, J.-F. and Czerucka, D. Saccharomyces Boulardii Modifies Salmonella Typhimurium Traffic and Host Immune Responses Along the Intestinal Tract. (2014) *PLoS ONE* **9**, 8: e103069.

Prosky, L., Asp, N.G., Schweizer, T.F., Devries, J.W. and Furda, I. Determination of Insoluble, Soluble, and Total Dietary Fiber in Foods and Food-Products - Interlaboratory Study. (1988) *Journal of the Association of Official Analytical Chemists* **71**, 5: 1017-1023.

Quast, C., Pruesse, E., Yilmaz, P., Gerken, J., Schweer, T., Yarza, P., Peplies, J. and Glockner, F.O. The Silva Ribosomal Rna Gene Database Project: Improved Data Processing and Web-Based Tools. (2013) *Nucleic Acids Research* **41**, Database issue: D590-6.

Raffatellu, M., Wilson, R.P., Chessa, D., Andrews-Polymenis, H., Tran, Q.T., Lawhon, S., Khare, S., Adams, L.G. and Baumler, A.J. *Sipa*, *Sopa*, *Sopb*, *Sopd*, and *Sope2* Contribute to *Salmonella Enterica* Serotype Typhimurium Invasion of Epithelial Cells. (2005) *Infection and Immunity* **73**, 1: 146-154.

Ranjitkar, S., Lawley, B., Tannock, G. and Engberg, R.M. Bacterial Succession in the Broiler Gastrointestinal Tract. (2016) *Appl Environ Microbiol* **82**, 8: 2399-410.

Rastall, R.A. and Maitin, V. Prebiotics and Synbiotics: Towards the Next Generation. (2002) *Current Opinion in Biotechnology* **13**, 5: 490-496.

Rebellato, A.P., Bussi, J., Silva, J.G.S., Greiner, R., Steel, C.J. and Pallone, J.A.L. Effect of Different Iron Compounds on Rheological and Technological Parameters as Well as Bioaccessibility of Minerals in Whole Wheat Bread. (2017) *Food Research International* **94**: 65-71.

Rehman, H.U., Vahjen, W., Awad, W.A. and Zentek, J. Indigenous Bacteria and Bacterial Metabolic Products in the Gastrointestinal Tract of Broiler Chickens. (2007) *Arch Anim Nutr* **61**, 5: 319-35.

Rescigno, M., Rotta, G., Valzasina, B. and Ricciardi-Castagnoli, P. Dendritic Cells Shuttle Microbes across Gut Epithelial Monolayers. (2001) *Immunobiology* **204**, 5: 572-581.

Rincon, M.T., Ding, S.Y., McCrae, S.I., Martin, J.C., Aurilia, V., Lamed, R., Shoham, Y., Bayer, E.A. and Flint, H.J. Novel Organization and Divergent Dockerin Specificities in the Cellulosome System of Ruminococcus Flavefaciens. (2003) *Journal of Bacteriology* **185**, 3: 703-713.

Riviere, A., Moens, F., Selak, M., Maes, D., Weckx, S. and Vuyst, L.D. The Ability of Bifidobacteria to Degrade Arabinoxylan Oligosaccharide Constituents and Derived Oligosaccharides Is Strain Dependent. (2014) *Applied and Environmental Microbiology* **80**, 1: 204-217.

Rogowski, A., Baslé, A., Farinas, C.S., Solovyova, A., Mortimer, J.C., Dupree, P., Gilbert, H.J. and Bolam, D.N. Evidence That Gh115 A-Glucuronidase Activity, Which Is Required to Degrade Plant Biomass, Is Dependent on Conformational Flexibility. (2014) *The Journal of Biological Chemistry* **289**, 1: 53-64.

Sakata, T. and von Engelhardt, W. Stimulatory Effect of Short Chain Fatty Acids on the Epithelial Cell Proliferation in Rat Large Intestine. (1983) *Comp Biochem Physiol A Comp Physiol* **74**, 2: 459-62.

Saki, A.A., Matin, H.R.H., Zamani, P., Tabatabai, M.M. and Vatanchian, M. Various Ratios of Pectin to Cellulose Affect Intestinal Morphology, DNA Quantitation, and Performance of Broiler Chickens. (2011) *Livestock Science* **139**, 3: 237-244.

Sanchez, B., Lopez, P., Gonzalez-Rodriguez, I., Suarez, A., Margolles, A. and Urdaci, M.C. A Flagellin-Producing Lactococcus Strain: Interactions with Mucin and Enteropathogens. (2011) *Fems Microbiology Letters* **318**, 2: 101-107.

Sassone-Corsi, M., Nuccio, S.P., Liu, H., Hernandez, D., Vu, C.T., Takahashi, A.A., Edwards, R.A. and Raffatellu, M. Microcins Mediate Competition among Enterobacteriaceae in the Inflamed Gut. (2016) *Nature* **540**, 7632: 280-283.

Saulnier, L., Sado, P.E., Branlard, G., Charmet, G. and Guillon, F. Wheat Arabinoxylans: Exploiting Variation in Amount and Composition to Develop Enhanced Varieties. (2007) *Journal of Cereal Science* **46**, 3: 261-281.

Schamberger, G.P. and Diez-Gonzalez, F. Characterization of Colicinogenic Escherichia Coli Strains Inhibitory to Enterohemorrhagic Escherichia Coli. (2004) *J Food Prot* **67**, 3: 486-92.

Scott, K.P., Martin, J.C., Duncan, S.H. and Flint, H.J. Prebiotic Stimulation of Human Colonic Butyrate-Producing Bacteria and Bifidobacteria, in Vitro. (2014) *FEMS Microbiol Ecol* **87**, 1: 30-40.

Sergeant, M.J., Constantinidou, C., Cogan, T.A., Bedford, M.R., Penn, C.W. and Pallen, M.J. Extensive Microbial and Functional Diversity within the Chicken Cecal Microbiome. (2014) *Plos One* **9**, 3.

Shaufi, M.A.M., Sieo, C.C., Chong, C.W., Gan, H.M. and Ho, Y.W. Deciphering Chicken Gut Microbial Dynamics Based on High-Throughput 16s Rrna Metagenomics Analyses. (2015) *Gut Pathogens* **7**.

Shin, R., Suzuki, M. and Morishita, Y. Influence of Intestinal Anaerobes and Organic Acids on the Growth of Enterohaemorrhagic Escherichia Coli O157:H7. (2002) *Journal of Medical Microbiology* **51**, 3: 201-6.

Shinkai, T. and Kobayashi, Y. Localization of Ruminal Cellulolytic Bacteria on Plant Fibrous Materials as Determined by Fluorescence in Situ Hybridization and Real-Time Pcr. (2007) *Applied and Environmental Microbiology* **73**, 5: 1646-52.

Simpson, H.L. and Campbell, B.J. Review Article: Dietary Fibre-Microbiota Interactions. (2015) *Alimentary Pharmacology & Therapeutics* **42**, 2: 158-179.

Singh, P. and Gill, P.K. Production of Inulinases: Recent Advances. (2006) *Food Technology and Biotechnology* **44**, 2: 151-162.

Sonnenburg, E.D. and Sonnenburg, J.L. Starving Our Microbial Self: The Deleterious Consequences of a Diet Deficient in Microbiota-Accessible Carbohydrates. (2014) *Cell Metabolism* **20**, 5: 779-786.

Spring, P., Wenk, C., Dawson, K.A. and Newman, K.E. The Effects of Dietary Mannanoligosaccharides on Cecal Parameters and the Concentrations of Enteric Bacteria in the Ceca of *Salmonella*-Challenged Broiler Chicks. (2000a) *Poultry Science* **79**, 2: 205-211.

Spring, P., Wenk, C., Dawson, K.A. and Newman, K.E. The Effects of Dietary Mannaoligosaccharides on Cecal Parameters and the Concentrations of Enteric Bacteria in the Ceca of Salmonella-Challenged Broiler Chicks. (2000b) *Poultry Science* **79**, 2: 205-11.

Stanley, D., Geier, M.S., Hughes, R.J., Denman, S.E. and Moore, R.J. Highly Variable Microbiota Development in the Chicken Gastrointestinal Tract. (2013) *PLoS One* **8**, 12: e84290.

Stanley, D., Hughes, R.J. and Moore, R.J. Microbiota of the Chicken Gastrointestinal Tract: Influence on Health, Productivity and Disease. (2014) *Applied Microbiology and Biotechnology* **98**, 10: 4301-10.

Stevenson, L., Phillips, F., O'Sullivan, K. and Walton, J. Wheat Bran: Its Composition and Benefits to Health, a European Perspective. (2012) *International Journal of Food Sciences and Nutrition* **63**, 8: 1001-1013.

Stewart, M.L. and Slavin, J.L. Particle Size and Fraction of Wheat Bran Influence Short-Chain Fatty Acid Production *in Vitro*. (2009) *British Journal of Nutrition* **102**, 10: 1404-7.

Surget, A. and Barron, C. Histologie Du Grain De Blé. (2005) *Industries des céréales* **145**: 3-7.

Svihus, B. The Gizzard: Function, Influence of Diet Structure and Effects on Nutrient Availability. (2011) *Worlds Poultry Science Journal* **67**, 2: 207-223.

Svihus, B. Function of the Digestive System. (2014) *Journal of Applied Poultry Research* **23**, 2: 306-314.

Tappenden, K.A. and McBurney, M.I. Systemic Short-Chain Fatty Acids Rapidly Alter Gastrointestinal Structure, Function, and Expression of Early Response Genes. (1998) *Digestive Diseases and Sciences* **43**, 7: 1526-1536.

Teirlynck, E., Bjerrum, L., Eeckhaut, V., Huygebaert, G., Pasmans, F., Haesebrouck, F., Dewulf, J., Ducatelle, R. and Van Immerseel, F. The Cereal Type in Feed Influences Gut Wall Morphology and Intestinal Immune Cell Infiltration in Broiler Chickens. (2009a) *Br J Nutr* **102**, 10: 1453-61.

Teirlynck, E., Haesebrouck, F., Pasmans, F., Dewulf, J., Ducatelle, R. and Van Immerseel, F. The Cereal Type in Feed Influences Salmonella Enteritidis Colonization in Broilers. (2009b) *Poult Sci* **88**, 10: 2108-12.

Tellez, G., Dean, C.E., Corrier, D.E., Deloach, J.R., Jaeger, L. and Hargis, B.M. Effect of Dietary Lactose on Cecal Morphology, Ph, Organic-Acids, and *Salmonella*-Enteritidis Organ Invasion in Leghorn Chicks. (1993) *Poultry Science* **72**, 4: 636-642.

Theander, O., Aman, P., Westerlund, E., Andersson, R. and Pettersson, D. Total Dietary Fiber Determined as Neutral Sugar Residues, Uronic Acid Residues, and Klason Lignin (the Uppsala Method): Collaborative Study. (1995) *J AOAC Int* **78**, 4: 1030-44.

Thulesen, J., Hartmann, B., Holst, J.J. and Poulsen, S.S. Diabetic Intestinal Growth Adaptation and Glucagon-Like Peptide 2 (Glp-2) in the Rat. Effects of Dietary Fibers. (1999) *Gastroenterology* **116**, 4: A581-A581.

Topping, D.L. and Clifton, P.M. Short-Chain Fatty Acids and Human Colonic Function: Roles of Resistant Starch and Nonstarch Polysaccharides. (2001) *Physiological Reviews* **81**, 3: 1031-1064.

Truchado, P., Van den Abbeele, P., Riviere, A., Possemiers, S., De Vuyst, L. and Van de Wiele, T. *Bifidobacterium Longum* D2 Enhances Microbial Degradation of Long-Chain Arabinoxylans in an in Vitro Model of the Proximal Colon. (2015) *Beneficial Microbes* **6**, 6: 849-860.

Tungland, B.C. Fructo-Oligosaccharides and Other Fructans: Structures and Occurrences, Production, Regulatory Aspects, Food Applications and Nutritional Health Significance. (2002) *Abstracts of Papers of the American Chemical Society* **223**: U105-U105.

Ubeda, C., Djukovic, A. and Isaac, S. Roles of the Intestinal Microbiota in Pathogen Protection. (2017) *Clin Transl Immunology* **6**, 2: e128.

Vaishnava, S., Behrendt, C.L., Ismail, A.S., Eckmann, L. and Hooper, L.V. Paneth Cells Directly Sense Gut Commensals and Maintain Homeostasis at the Intestinal Host-Microbial Interface. (2008) *Proc Natl Acad Sci U S A* **105**, 52: 20858-63.

van Asten, A. and van Dijk, J.E. Distribution of "Classic" Virulence Factors among Salmonella Spp. (2005) *Fems Immunology and Medical Microbiology* **44**, 3: 251-259.

van de Wiele, T., Boon, N., Possemiers, S., Jacobs, H. and Verstraete, W. Inulin-Type Fructans of Longer Degree of Polymerization Exert More Pronounced in Vitro Prebiotic Effects. (2007) *J Appl Microbiol* **102**, 2: 452-60.

Van den Abbeele, P., Gerard, P., Rabot, S., Bruneau, A., El Aidy, S., Derrien, M., Kleerebezem, M., Zoetendal, E.G., Smidt, H., Verstraete, W., Van de Wiele, T. and Possemiers, S. Arabinoxylans and Inulin Differentially Modulate the Mucosal and Luminal Gut Microbiota and Mucin-Degradation in Humanized Rats. (2011) *Environ Microbiol* **13**, 10: 2667-80.

van den Broek, L.A.M., Lloyd, R.M., Beldman, G., Verdoes, J.C., McCleary, B.V. and Voragen, A.G.J. Cloning and Characterization of Arabinoxylan Arabinofuranohydrolase-D3 (Axhd3) from Bifidobacterium Adolescentis Dsm20083. (2005) *Applied Microbiology and Biotechnology* **67**, 5: 641-647.

van der Wielen, P.W., Keuzenkamp, D.A., Lipman, L.J., van Knapen, F. and Biesterveld, S. Spatial and Temporal Variation of the Intestinal Bacterial Community in Commercially Raised Broiler Chickens During Growth. (2002) *Microb Ecol* **44**, 3: 286-93.

Van Immerseel, F., De Buck, J., Boyen, F., Bohez, L., Pasmans, F., Volf, J., Sevcik, M., Rychlik, I., Haesebrouck, F. and Ducatelle, R. Medium-Chain Fatty Acids Decrease Colonization and Invasion through *Hila* Suppression Shortly after Infection of Chickens with *Salmonella Enterica* Serovar Enteritidis. (2004) *Applied and Environmental Microbiology* **70**, 6: 3582-3587.

Van Immerseel, F., De Zutter, L., Houf, K., Pasmans, F., Haesebrouck, F. and Ducatelle, R. Strategies to Control *Salmonella* in the Broiler Production Chain. (2009) *Worlds Poultry Science Journal* **65**, 3: 367-391.

Van Immerseel, F., Russell, J.B., Flythe, M.D., Gantois, I., Timbermont, L., Pasmans, F., Haesebrouck, F. and Ducatelle, R. The Use of Organic Acids to Combat *Salmonella* in Poultry: A Mechanistic Explanation of the Efficacy. (2006) *Avian Pathology* **35**, 3: 182-8.

Van Soest, P.J. Use of Detergents in the Analysis of Fibrous Feeds. Ii. Determination of Plant Cell-Wall Constituents. (1963) *Journal of the Association of Official Analytical Chemists* **48**: 829-835.

Van Soest, P.J. and Wine, R.H. Use of Detergents in the Analysis of Fibrous Feeds. Iv. Determination of Plant Cell-Wall Constituents. (1967) *Journal of the Association of Official Analytical Chemists* **50**: 50-55.

Vandeplas, S., Dauphin, R.D., Beckers, Y., Thonart, P. and Thewis, A. *Salmonella* in Chicken: Current and Developing Strategies to Reduce Contamination at Farm Level. (2010) *Journal of Food Protection* **73**, 4: 774-785.

Vardakou, M., Palop, C.N., Gasson, M., Narbad, A. and Christakopoulos, P. In Vitro Three-Stage Continuous Fermentation of Wheat Arabinoxylan Fractions and Induction of Hydrolase Activity by the Gut Microflora. (2007) *International Journal of Biological Macromolecules* **41**, 5: 584-589.

Velge, P., Wiedemann, A., Rosselin, M., Abed, N., Boumart, Z., Chausse, A.M., Grepinet, O., Namdari, F., Roche, S.M., Rossignol, A. and Virlogeux-Payant, I. Multiplicity of *Salmonella* Entry Mechanisms, a New Paradigm for *Salmonella* Pathogenesis. (2012) *Microbiologyopen* **1**, 3: 243-258.

Verspreet, J., Damen, B., Broekaert, W.F., Verbeke, K., Delcour, J.A. and Courtin, C.M. A Critical Look at Prebiotics within the Dietary Fiber Concept. (2016) *Annual Review of Food Science and Technology* **7**: 167-90.

Verspreet, J., Dornez, E., Van den Ende, W., Delcour, J.A. and Courtin, C.M. Cereal Grain Fructans: Structure, Variability and Potential Health Effects. (2015a) *Trends in Food Science & Technology* **43**, 1: 32-42.

Verspreet, J., Hansen, A.H., Dornez, E., Delcour, J.A., Van den Ende, W., Harrison, S.J. and Courtin, C.M. Lc-Ms Analysis Reveals the Presence of Graminan- and Neo-Type Fructans in Wheat Grains. (2015b) *Journal of Cereal Science* **61**: 133-138.

Wachtershauser, A., Loitsch, S.M. and Stein, J. Ppar-Gamma Is Selectively Upregulated in Caco-2 Cells by Butyrate. (2000) *Biochemical and Biophysical Research Communications* **272**, 2: 380-385.

Waite, D.W. and Taylor, M.W. Characterizing the Avian Gut Microbiota: Membership, Driving Influences, and Potential Function. (2014) *Frontiers in Microbiology* **5**.

Walker, A.W., Duncan, S.H., Harmsen, H.J., Holtrop, G., Welling, G.W. and Flint, H.J. The Species Composition of the Human Intestinal Microbiota Differs between Particle-Associated and Liquid Phase Communities. (2008) *Environmental Microbiology* **10**, 12: 3275-83.

Wang, H., Wang, P., Wang, X., Wan, Y. and Liu, Y. Butyrate Enhances Intestinal Epithelial Barrier Function Via up-Regulation of Tight Junction Protein Claudin-1 Transcription. (2012) *Digestive Diseases and Sciences* **57**, 12: 3126-3135.

Wang, L., Li, J., Li, J., Jr., Li, R.X., Lv, C.F., Li, S., Mi, Y.L. and Zhang, C.Q. Identification of the Paneth Cells in Chicken Small Intestine. (2016) *Poult Sci* **95**, 7: 1631-5.

Wang, Q., Cui, Y., Wang, W., Xu, J. and Xu, L. Production of Two Bacteriocins in Various Growth Conditions Produced by Gram-Positive Bacteria Isolated from Chicken Cecum. (2012) *Can J Microbiol* **58**, 1: 93-101.

Wei, S., Morrison, M. and Yu, Z. Bacterial Census of Poultry Intestinal Microbiome. (2013) *Poultry Science* **92**, 3: 671-683.

Williams, J., Mallet, S., Leconte, M., Lessire, M. and Gabriel, I. The Effects of Fructo-Oligosaccharides or Whole Wheat on the Performance and Digestive Tract of Broiler Chickens. (2008) *British Poultry Science* **49**, 3: 329-339.

Winnen, B., Schlumberger, M.C., Sturm, A., Schupbach, K., Siebenmann, S., Jenny, P. and Hardt, W.D. Hierarchical Effector Protein Transport by the Salmonella Typhimurium Spi-1 Type Iii Secretion System. (2008) *Plos One* **3**, 5.

Wolfe, A.J. "Glycolysis for Microbiome Generation." In *Metabolism and Bacterial Pathogenesis*: American Society of Microbiology, 2015.

Wood, P.J. Oat and Rye Beta-Glucan: Properties and Function. (2010) *Cereal Chemistry* **87**, 4: 315-330.

Xiao, Y.P., Xiang, Y., Zhou, W.D., Chen, J.G., Li, K.F. and Yang, H. Microbial Community Mapping in Intestinal Tract of Broiler Chicken. (2017) *Poultry Science* **96**, 5: 1387-1393.

Xu, J., Bjursell, M.K., Himrod, J., Deng, S., Carmichael, L.K., Chiang, H.C., Hooper, L.V. and Gordon, J.I. A Genomic View of the Human-*Bacteroides Thetaiotaomicron* Symbiosis. (2003) *Science* **299**, 5615: 2074-2076.

Yacoubi, N., Van Immerseel, F., Ducatelle, R., Rhayat, L., Bonnin, E. and Saulnier, L. Water-Soluble Fractions Obtained by Enzymatic Treatment of Wheat Grains Promote Short Chain Fatty Acids Production by Broiler Cecal Microbiota. (2016) *Animal Feed Science and Technology* **218**: 110-119.

Yang, G.Q., Yin, Y., Liu, H.Y. and Liu, G.H. Effects of Dietary Oligosaccharide Supplementation on Growth Performance, Concentrations of the Major Odor-Causing Compounds in Excreta, and the Cecal Microflora of Broilers. (2016) *Poultry Science* **95**, 10: 2342-2351.

Zhang, Y.-J., Li, S., Gan, R.-Y., Zhou, T., Xu, D.-P. and Li, H.-B. Impacts of Gut Bacteria on Human Health and Diseases. (2015) *International Journal of Molecular Sciences* **16**, 4: 7493-7519.

PART 2

SCIENTIFIC AIMS

SCIENTIFIC AIMS

The crucial role of the microbiota in the gastrointestinal tract for host well-being and health is well-recognized. There is an increasing awareness of society and feed/food industry for the possibility and need to promote host well-being through healthy and functional high quality food and feed. For a while already, the search for appropriate feed additives that can improve the composition and activity of the intestinal microbial community is ongoing. Several prebiotic compounds, mostly purified oligosaccharides, have been proposed as suitable candidates. AXOS derived from wheat bran, for example, have been shown to induce higher cecal counts of bifidobacteria when supplemented to the feed of broilers (Courtin et al. 2008). Moreover, these AXOS could also reduce cecal counts of *Salmonella* Enteritidis bacteria in a *Salmonella* infection model (Eeckhaut et al. 2008). The main drawback of working with purified oligosaccharides is that they are expensive to manufacture: insoluble dietary fibres may cost up to 2000 €/ton, while prices for soluble dietary fibres can go up to 3000 €/ton.

Wheat bran is a byproduct of the milling of wheat into flour and is available in large quantities in Europe, therefore it is relatively cheap (~150 €/ton). It is the most concentrated source of insoluble dietary fibre in the Western diet and consists of both fermentable and non-fermentable fibres. It is a well-recognized healthy food and feed constituent; EFSA recently approved two health claims related to beneficial physiological effects (EFSA 2010). In addition, wheat bran can be easily technically modified and since technologies employed to alter wheat bran are becoming increasingly available to the industry, one can envisage modifying wheat bran properties to yield an optimal impact on the gut microbiota. At this point nothing is known about the influence of technical modification of the substrate on the composition of the adherent community. Simple modifications such as particle size reductions, could alter the bran structure and affect the colonizing species and would provide an interesting and relatively simple tool to steer the adherent community in a beneficial manner.

The **general aim** of this thesis was to investigate whether technically modified wheat bran could be a valuable tool to induce beneficial shifts in the cecal community composition of broilers and potentially improve their overall health.
More specifically the aim of the **first study** was to investigate whether different technically modified wheat bran fractions could function as dietary platform, supporting the attachment and metabolic activity of specific members of the cecal microbiome.

The aim of the **second study** was to investigate whether the application of wheat bran reduced in particle size to a different degree could protect against cecal pathogen colonization, using *Salmonella* as a model challenge organism.

In the **third study** we aimed to provide a proof of concept related to the previous chapters. We tested whether the results obtained by supplementing wheat bran to the feed of *Salmonella* challenged broilers could be mimicked by delivering two members of the bacterial community which were specifically attached to wheat bran.

REFERENCE LIST

Courtin, C.M., Swennen, K., Broekaert, W.F., Swennen, Q., Buyse, J., Decuypere, E., Michiels, C.W., Ketelaere, B.D. and Delcour, J.A. Effects of Dietary Inclusion of Xylooligosaccharides, Arabinoxylooligoscaccharides and Soluble Arabinoxylan on the Microbial Composition of Caecal Contents of Chickens. (2008) *Journal of the Science of Food and Agriculture* **88**, 14: 2517-2522.

Eeckhaut, V., Van Immerseel, F., Dewulf, J., Pasmans, F., Haesebrouck, F., Ducatelle, R., Courtin, C.M., Delcour, J.A. and Broekaert, W.F. Arabinoxylooligosaccharides from Wheat Bran Inhibit *Salmonella* Colonization in Broiler Chickens. (2008) *Poultry Science* **87**, 11: 2329-34.

EFSA. Scientific Opinion on the Substantiation of Health Claims Related to Wheat Bran Fibre and Increase in Faecal Bulk (Id 3066), Reduction in Intestinal Transit Time (Id 828, 839, 3067, 4699) and Contribution to the Maintenance or Achievement of a Normal Body Weight (Id 829) Pursuant to Article 13(1) of Regulation (Ec) No 1924/2006. (2010) *EFSA Journal* **8**, 10: 1817-n/a.

1 • Reduced particle size wheat bran is colonized by a butyrogenic community and alters the cecal microbiota composition of broilers

2 • Reduced particle size wheat bran is butyrogenic and lowers *Salmonella* colonization when added to poultry feed

3 • Oral administration of *Ruminococcus torques* and *Bifidobacterium pseudolongum* increases resilience against *Salmonella* infections in broilers

PART 3

EXPERIMENTAL STUDIES

1

REDUCED PARTICLE SIZE WHEAT BRAN IS COLONIZED BY A BUTYROGENIC COMMUNITY AND ALTERS THE CECAL MICROBIOTA COMPOSITION OF BROILERS

Vermeulen K., Verspreet J., Courtin C.M., Haesebrouck F., Baeyen S., Haegeman A., Ducatelle R., Van Immerseel F. (submitted at Microbiome)

ABSTRACT

Dietary fibres are known to improve gastrointestinal health of both humans and animals in many different ways. They can increase the bulking capacity, improve transit times and, depending on the fibre, even stimulate the growth and activity of resident beneficial bacteria. Wheat bran is a readily available byproduct of flour processing and is a highly concentrated source of (in)soluble dietary fibre. In the current study we investigated whether reducing the particle size of wheat bran affected the colonizing microbial community using batch fermentations with cecal inoculum from seven different chickens. We also investigated the effect of in-feed administration of regular wheat bran (1690 µm) and wheat bran with reduced particle size (280 µm) on the cecal microbial community composition of broilers. During batch fermentation, particle size reduced wheat bran was colonized by a butyrogenic community, mainly consisting of *Lachnospiraceae* species. A co-occurring enrichment of bifidobacteria and lactobacilli can hypothetically sustain this group of butyrate producers through cross-feeding on lactate. The relative abundance of the *Enterobacteriaceae* decreased in the particle-associated community for both regular and particle size reduced bran compared to the free-living community. In addition, the community attached to wheat bran was enriched in xylan degrading bacteria. When administered as a feed additive to broilers, wheat bran with reduced particle size significantly increased the richness of the cecal microbiota. It also increased the abundance of butyrate producing bacteria and decreased abundance of members of the *Enterobacteriaceae* family in the ceca. Particle size reduction of wheat bran thus resulted in colonization of the bran particles by a very specific community and can be used to steer towards a beneficial microbiota when administered as feed additive to broilers.

1. INTRODUCTION

The greatest determinant of the gut microbiota composition, in addition to age and breed, is diet. Dietary levels and quality of fat, protein and carbohydrates, and the use of exogenous feed enzymes all impact the gut microbiota (Geier et al. 2009, Torok et al. 2013, De Maesschalck et al. 2015a). Dietary fibres (DFs) are carbohydrate polymers, including lignin and plant associated substances, that escape digestion in the small intestine and pass into the hindgut where they are (partially) fermented by the microbiota (Codex Alimentarius Commission 2009, Howlett et al. 2010). They can be classified as either being soluble or insoluble (Kumar et al. 2012). Most water soluble DF (such as pectins) are fermentable, while insoluble DF (such as cellulose, lignin and hemicellulose) are thought to be less fermentable (Johnson 2001, Stewart & Slavin 2009, Verspreet et al. 2016). Certain DF are known to improve gastrointestinal health of both humans and animals by stimulating the growth of resident beneficial bacteria (Crittenden et al. 2002, Biggs & Parsons 2005, Courtin et al. 2008, Damen et al. 2011).

Wheat bran, a by-product of the dry milling of wheat grains into flour, is a highly concentrated source of DF. It consists of the outer layers (cuticle, pericarp and seed coat) of the wheat kernel retaining only small amounts of aleurone and starchy endosperm. Wheat bran as such consists of both fermentable (starch, protein, arabinoxylans with low substitution degrees) and non-fermentable (cellulose, lignin, arabinoxylans with high substitution degrees) components (Maes & Delcour 2001, 2002, Leitch et al. 2007, Hemdane et al. 2016). The polysaccharide fraction consists mainly of insoluble DF while only a small proportion is soluble (Maes & Delcour 2001, 2002). Within the intestinal environment the insoluble fraction shows great resemblance with (in)organic macroaggregates in aquatic ecosystems. It has been shown that these aggregates are heavily colonized by bacteria and other heterotrophic microorganisms (Simon et al. 2002). Several studies have suggested that bacterial community structures are dissimilar between particle-associated and free-living bacteria, the former exhibiting higher enzymatic activity than the latter (Simon et al. 2002). It is evident that these aggregates and their surroundings are hot-spots of microbial processes, with the plume of the solutes leaking out of the aggregates (Simon et al. 2002). In accordance, several studies have shown that particulate matter in the intestine harbors a unique and distinct bacterial community which differs from the free-living community (Leitch et al. 2007, Walker et al. 2008, De Paepe et al. 2017). The particle-associated community composition seems to depend strongly on the

substrate (Leitch et al. 2007, Walker et al. 2008). While no such data is available for poultry, in ruminants, it has been shown that specific ruminal bacteria develop a dynamic biofilm upon digesta particles and possess higher fibrolytic activity than the luminal population (Michalet-Doreau et al. 2001, Larue et al. 2005, Shinkai & Kobayashi 2007). With regard to wheat bran, it has recently been shown in an *in vitro* study that it can be colonized by a specific population of bacteria from the human gut microbiome (De Paepe et al. 2017). Attached to wheat bran, the close proximity between the different bacteria could foster an enhanced cross-feeding and more efficient use of substrate and thus more elaborate energy harvest. It is hitherto unknown whether the size of the particles matters with regard to colonization patterns. In this study we examined whether wheat bran with a reduced particle size in comparison to unmodified wheat bran is colonized by a specific bacterial community and whether these bran particles can induce shifts in the composition of the cecal microbiota when supplemented to the feed of broilers.

2. MATERIALS AND METHODS

2.1 MODIFIED WHEAT BRAN FRACTIONS

The particle size of wheat bran was reduced as described previously (Jacobs et al. 2015). In short, commercial wheat bran (Dossche Mills, Deinze, Belgium) was reduced in particle size with a Cyclotec 1093 Sample mill (FOSS, Höganäs, Sweden). By changing the grinding ring and/or the mesh size of the final sieve of the mill, bran fractions with different particle sizes were obtained. The particle size distribution of the resulting fractions was determined by sieving 20.0 g of bran on a set of sieves with mesh sizes of 4500, 2000, 1000, 710, 500, 400, 250, 200, 160, 125, 112, 90, 50, and 38 µm. The set of sieves was shaken for 30 min at a frequency of 1.5 s^{-1} with a retch Vibratory Sieve Shaker (Aartselaar, Belgium) after which each sieve was gently brushed to avoid clogging of the sieve pores. The mass of the bran that remained on each sieve was determined and used to calculate a mass based average particle size:

$$d_{av} = \sum d_i \, . \, m_i$$

with d_i = (upper size limit of bran fraction i + lower limit of bran fraction i)/2 and m_i = mass fraction, i.e. mass on sieve i/sum of masses of all fractions.

Regular wheat bran (1690 µm) and wheat bran with an average reduced particle size of 280 µm, hereafter referred to as WB and WB280 respectively, were used in the experiments described below.

2.2 *IN VITRO* DIGESTION AND FERMENTATION

Different wheat bran fractions (WB and WB280) were predigested *in vitro* using a protocol based on Wu et al. (2004) which was slightly adapted (Wu et al. 2004). First, 1 g substrate was incubated with 1.5 ml 0.03 M HCl (40°C for 30 min) to mimic the initial stages of digestion in the crop. Secondly, the digestion in the proventriculus and gizzard was simulated by incubating the substrate with 3000 U of pepsin from porcine gastric mucosa (Sigma-Aldrich, St. Louis, United States) in 1 ml 1.5 M HCl (40°C for 45 min). To simulate digestion in the duodenum, 1 ml 1 M $NaHCO_3$ and 3.7 mg pancreatin from porcine pancreas (Sigma-Aldrich) were added and incubated for 2 h at 40°C. Subsequently the predigested bran fractions were

centrifuged (5 min, 5000xg) and washed twice with 20 ml of aqua dest. The resulting pellet was retained and lyophilized. The *in vitro* fermentations were performed using a nutrient-poor medium described by Moura et al. (2007) with minor modifications, previously described by De Maesschalck et al. (2015) (Moura et al. 2007, De Maesschalck et al. 2015b). The pH of the medium was adjusted to 6.5. The ceca of 4-week old Ross 308 broilers were isolated and brought immediately into an anaerobic cabinet (Ruskinn technology, Bridgend, United Kingdom) with 84% N_2, 8% H_2 and 8% CO_2 at 37°C. The cecal content of an individual broiler, diluted 1000-fold, was used as inoculum. The lyophilized predigested fractions were supplemented to the nutrient-poor medium at a concentration of 1% (w/v). Non-supplemented medium was used as a control (hereafter referred to as control or non-attached community). After 24 h anaerobic incubation at 37°C, the bran fraction was collected by centrifugation (5 min, 700xg). To remove all non- and loosely attached bacteria, the bran fractions were washed using a protocol previously described by Leitch et al. (2007) and slightly adapted by De Paepe et al. (2017) (Leitch et al. 2007, De Paepe et al. 2017). The experiment was performed seven times using cecal contents obtained from seven different chickens.

2.3 *IN VIVO* EXPERIMENT

Thirty one-day-old Ross 308 broilers were obtained from a local hatchery and housed in three pens (10 chickens per pen) on wood shavings. A commercial standard wheat/rye-based feed and drinking water were provided *ad libitum*. Group one received no supplements. The feed of group two and three was supplemented respectively with 1% WB and 1% WB280. The chickens were euthanized when they were ten days old and cecal content was collected for microbiota analysis.

2.4 DNA EXTRACTION

DNA was extracted from cecum content and wheat bran associated bacteria using the CTAB method as described previously by Griffiths et al. (2000) and Kowalchuk et al. (1999) (Kowalchuk et al. 1999, Griffiths et al. 2000). To 100 mg of cecal content or 100 mg of washed bran, 0.5 ml CTAB buffer (hexadecyltrimethylammonium bromide >98% (Sigma Aldrich) 5% (w/v), 0.35 M NaCl, 120 nM K_2HPO_4) and 0.5 ml phenol-chloroform-isoamyl alcohol (25:24:1) (Sigma-Aldrich) was added followed by homogenization in destruction tubes. The samples were

shaken 6 times for 30 seconds using a beadbeater (MagnaLyser, Roche, Basel, Switzerland) at 6000xg with 30 seconds in between shakings. After centrifugation (10 min, 8000xg), 300 µl of the supernatant was transferred to a new tube. Then, samples were re-extracted with 250 µl CTAB buffer. After homogenization, samples were centrifuged (10 min, 8000xg) and 300 µl of supernatant was added to the first 300 µl. Phenol was removed by adding an equal volume of chloroform-isoamyl alcohol (24:1) (Sigma-Aldrich). The aqueous phase was transferred to a new Eppendorf tube. Nucleic acids were precipitated with 2 volumes of PEG-6000 solution (polyethyleenglycol 30% (w/v), 1.6 M NaCl) for 2 hours at room temperature. After centrifugation (20 min, 13000xg), the pellet was rinsed with 1 ml of ice-cold, 70% (v/v) ethanol. The pellet was dried and resuspended in 100 µl RNAse free water (VWR, Leuven, Belgium).

2.5 16S rRNA AMPLICON SEQUENCING

The 16S rRNA sequencing using MiSeq v2 technology (2x250 bp) from Illumina was performed at the GenoToul Genomics and Transcriptomics facility (Auzeville, France). The primers used were 343F (5'CTTTCCCTACACGACGCTCTTCCGATCTACGGRAGGCAGCAG3') and 784R (5'GGAGTTCAGACGTGTGCTCTTCCGATCTTACCAGGGTATCTAATCCT3') targeting the hypervariable 16S rRNA V3-V4 region. The amplification mix contained 5 U of FastStart high fidelity polymerase (Roche Diagnostics, Vilvoorde, Belgium), 8 µl of dNTP mix 250 µM (Eurogentec, Liège, Belgium), 2 µl of each primer (20 µM) and 100 ng of genomic DNA in a volume of 100 µl. Thermocycling conditions consisted of a denaturation at 94°C for 2 min followed by 30 cycles at 94°C for 60 s, 65°C for 40 s, 72°C for 30 s and a final elongation step of 10 min at 72°C. These amplifications were performed on a Mastercycler Pro (Eppendorf, Hamburg, Germany). Subsequently, DNA was purified using HighPrep™ PCR (MagBio Genomics.Inc, Gaithersburg, USA) following the protocol of the manufacturer. Single multiplexing was performed using a 6-bp index, which was added during a second PCR with 12 cycles. PCR products were purified and the quality and the fragment length were checked using Agilent DNA 7500 DNA chip (Agilent Technologies, Santa Clara, USA) following the manufacturer's protocol. The resulting products were purified and loaded onto an Illumina MiSeq cartridge according to the manufacturer's instructions (Illumina Inc., San Diego, CA). The quality of the run was internally checked using control libraries generated from the PhiX virus (Illumina PhiX control; Illumina Inc.) as recommended by Illumina.

2.6 SEQUENCE PROCESSING

Demultiplexing of the amplicon dataset and deletion of the barcodes was done by the sequencing provider. Raw Illumina forward and reverse reads were also trimmed and merged by the sequencing provider. In the following steps, different programs of the Usearch software v7.0.1090 were used (Edgar 2013). Merged sequences were quality filtered with a maximum expected error of 3 with the "fastq_filter" option. Next, sequences of all samples that needed to be compared to each other were merged, dereplicated and sorted by size. In total 374,832 merged sequences were used for analysis, with a mean number of sequences per sample of 18,742. Clustering the reads into Operational Taxonomic Units (OTUs) was done using Uparse, with an identity level of 97% (Ihrmark et al. 2012, Edgar 2013). Chimeras were removed using "uchime_ref" with the RDP Gold database as a reference (Edgar et al. 2011). Finally, sequences of individual samples were mapped back to the representative OTUs using the "usearch_global" algorithm at 97% identity, and converted to an OTU table (McDonald et al. 2012). OTU tables of the 16S rRNA amplicon sequencing were analysed using the QIIME software package (v1.9.0) (Caporaso et al. 2010b). Taxonomy was assigned with the script "assign_taxonomy.py" using the uclust method considering maximum 3 database hits, with the Silva v119 97% rep set (as provided by QIIME) as reference. Representative bacterial OTU sequences were aligned to the SILVA 97% rep set using the PyNast algorithm with QIIME default parameters (Caporaso et al. 2010a, Quast et al. 2013). Rarefaction analysis was done using the "alpha_rarefaction.py" script and indicated that a sequencing depth of 10 000 reads is sufficient to analyze both the attached and planktonic communities *in vitro* and the bacterial community in the cecal samples from broilers.

2.7 DOWNSTREAM DATA PROCESSING AND STATISTICS

Multivariate analysis was done using the specific R package vegan (version 2.0–10) (Oksanen J 2010). The OTU tables were normalized by removing those OTUs with an abundance lower than 0.01% in all samples. Dissimilarity matrices (based on the Bray-Curtis dissimilarity index) were calculated from the OTU tables. Beta-diversity of the bacterial communities was studied by doing a PERMANOVA and a principal coordinate analysis (PCoA) on these dissimilarity indices.

To determine significant differences in composition of adhered and non-adhered communities, a Kruskall-Wallis test, followed by a Dunn's post hoc test was performed using SPSS Statistics 23 (IBM Corp, New York, United States).

Illumina data obtained from the *in vivo* trail were log transformed whereafter a one-way ANOVA was used, followed by a Tukey post hoc test to determine statistical differences in relative abundances of the different bacterial families (SPSS statistics 23, IBM Corp).

Functional differences based on bacterial 16S rRNA community composition were assessed with METAGENassist (Arndt et al. 2012). Input files were created in QIIME. OTUs were assigned, mapped and condensed into functional taxa, and filtered based on interquantile range after Pareto scaling. These data were then analyzed for 'metabolism phenotype', and Euclidian distance measure was used to visualize the results in a heatmap.

OTUs that were significantly enriched on bran material were identified on the species level using NCBI BLAST. For every hit a reference genome was chosen where after the NCBI protein databases was searched for the presence of xylan degradation enzymes, being, xylanases, feruloyl esterases, arabinofuranosidases, acetyl xylan esterases, glucuronidases and xylosidases.

2.8 QUANTITATIVE PCR FOR TOTAL BACTERIA AND BUTYRYL-COA:ACETATE-COA TRANSFERASE GENE

To quantify the number of total bacteria, primers Uni331F (5'TCCTACGGGAGGCAGCAGT3') and Uni797R (5'GGACTAACCAGGGTATCTAATCCTGTT3') were used (Hopkins et al. 2005). Amplification and detection was performed using the CFX384 BioRad detection system (BioRad, Nazareth-Eke, Belgium). Each reaction was run in triplicate in a 12µl total reaction mixture using 2x SensiMix™ SYBR No-ROX mix (Bioline, Kampenhout, Belgium), 0.5µM final primer concentration and 2µl of (50 ng/µl) DNA. The amplification program consisted of 1 cycle at 95°C for 10 min followed by 40 cycles of 1 min at 94°C, 1 min at 53°C and 2 min at 60°C. A melting curve analysis was done after amplification and was obtained by slow heating from 60°C to 95°C at a rate of 0.5°C/5 sec to confirm the specificity of the reaction. To quantify the number of gene copies encoding the butyryl-CoA:acetate-CoA transferase (CoAt) gene, primers BCoATscrF (5'GCIGAICATTTCACITGGAAYWS3') and BCoATscrR

(5'CCTGCCTTTGCAATRTCIACRAANGC3') were used (Louis & Flint 2007). The final primer concentration was 2.5µM and the amplification program consisted of 1 cycle at 95°C for 10 min followed by 40 cycles of 30 sec at 95°C, 30 sec at 53°C and 30 sec at 72°C. The relative number of *CoAt* gene copies in the samples was obtained by calculating the ratio of CoAt gene copies to total number of Bacteria for each sample. To determine significant differences in relative *CoAt* gene copies, a Kruskall-Wallis test was performed, followed by a Dunn's post hoc test. Statistical analysis was done with SPSS Statistics 23 (IBM Corp).

3. RESULTS

3.1 WHEAT BRAN PARTICLES OF DIFFERENT PARTICLE SIZE ARE COLONIZED BY A DISTINCTIVE MICROBIAL COMMUNITY

WB and WB280 were *in vitro* fermented using the cecal inoculum of seven broilers. After 24 h, all non- and loosely attached bacteria were removed by washing. Using a principal coordinate analysis, it was shown that the bacterial diversity of the attached community differed greatly from the non-attached community (control). Moreover, the attached microbial community was influenced by the particle size of the wheat bran particles (Figure 1A). The bacterial richness (number of observed OTUs) on both bran fractions was smaller as compared to that of the non-attached community (Figure 1B). Significant differences could be observed in relative abundances of specific 16S rRNA sequences at different taxonomic levels (Table S1, supplementary information).

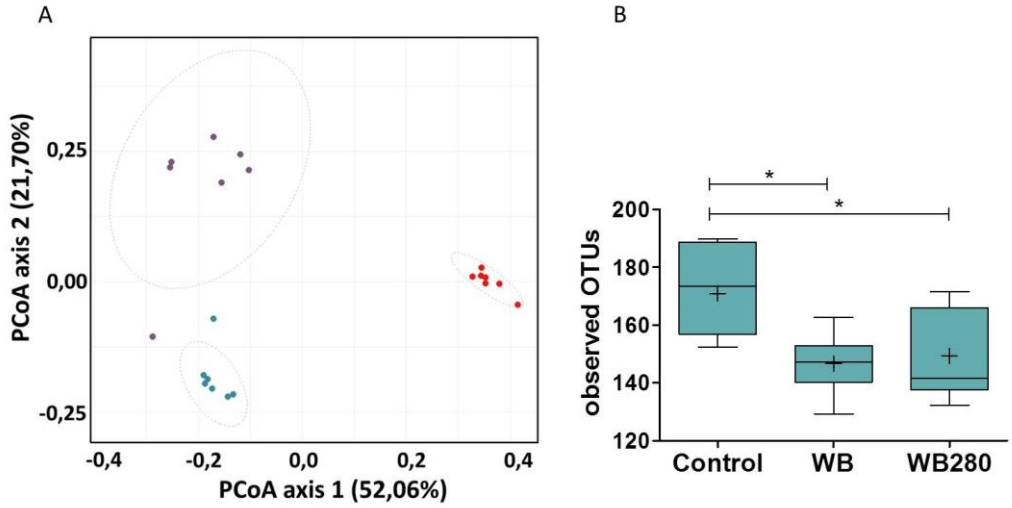


Figure 1 • Diversity and richness measures for the bacterial communities residing in the lumen, attached to WB and WB280. A PCoA biplot of the abundance based Bray-Curtis dissimilarity index revealed a distinctive microbial community attached to WB (●; n=7) and WB280 (●; n=7) or the non-attached community (control ●; n=7) (p=0.001) (A). Richness of the different bacterial communities, expressed as number of observed OTUs (B). Significant differences between WB, WB280 and control are indicated with *0.01<p<0.05; **0.001≤p≤0.01; ***p<0.001.

Members of the phylum **Actinobacteria** were significantly enriched on WB (34%) and WB280 (20%) compared to the control (4%, p<0.001 and p=0.039, respectively). Members belonging to **Bacteroidetes** preferred to attach to WB (8%) rather than to WB280 (3%, p=0.006) or to

reside in the planktonic fraction (3%, p=0.008). For WB280, 70% of the attached community belonged to **Firmicutes**, as opposed to only 46% for WB (p=0.004). **Proteobacteria** appeared to be reluctant to attach to bran material since their relative numbers decreased with 20% on WB and 29% on WB280 (p<0.001) compared to the control (Figure 2).

The following significant shifts in relative abundances on family and genus level could be observed: members of the ***Enterobacteriaceae*** family were relatively less abundant on the bran, and this was most evident for WB280 (3.5%) when compared to the control (27%, p<0.001). This shift in *Enterobacteriaceae* could be completely explained by the decrease in the relative abundance of the genus *Escherichia/Shigella* on WB280 compared to the control (p<0.001).

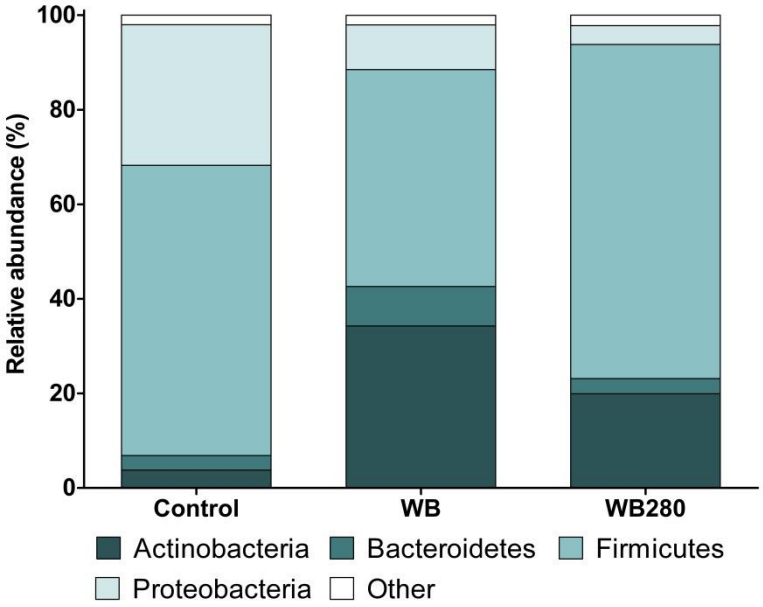


Figure 2 • Relative abundances of the most important bacterial phyla present in the non-attached community or attached to WB or WB280. Bran fractions were fermented in a nutrient poor medium with a chicken cecal inoculum for 24 h at 37°C. Bran was omitted in the control fermentations. Non- and loosely attached bacteria were removed by washing prior to DNA extraction.

Members of the ***Bacteroidaceae*** were enriched on WB when comparing relative abundances in the non-attached community and those in the community attached to WB280 (p=0.008, p=0.038 respectively). This shift fully coincided with the shifting relative numbers of members of the genus *Bacteroides* when comparing the control and WB280 with WB (p=0.07, p=0.043 respectively). Also, WB was colonized to a higher extent than WB280 by members of the ***Porphyromadaceae*** family (p=0.048) and the hereto belonging genus *Parabacteroides* (p=0.030). Relative abundances of ***Bifidobacteriaceae*** members were significantly increased on WB (34%) and WB280 (20%) compared to the control (4%) (WB p<0.001, WB280 p=0.039). This shift could be partly explained by an enrichment of the

genus *Bifidobacterium* on WB compared to the control (p=0.001). Two important families of butyrate producing bacteria showed significant shifts in their relative abundances. First, members of the ***Lachnospiraceae*** were enriched on WB280 (44%) compared to WB (15%) (p=0.001). This could mainly be explained by a relative increase of a group of uncultured *Lachnospiraceae* and *Lachnospiraceae* that could not be assigned to a specific genus within the family (WB 12%, WB280 41%; p<0.001). Members from the genus *Roseburia* show a ten-fold increase on WB280 when compared to the non-attached community (p=0.048). Compared to the control, no such enrichment of *Lachnospiraceae* could be observed for WB280 (p=0.311). Secondly, members of the ***Ruminococcaceae*** showed an opposite trend: they appeared to prefer WB (22%) or to occur non-attached (23%) and were significantly reduced on WB280 (14%, p=0.034, p=0.005, respectively). The most important genera contributing to this difference were the genus *Anaerotruncus*, a group of uncultured *Ruminococcaceae* and a group of OTUs that could not be assigned to a specific genus within the *Ruminococcaceae* family. Members of the genus *Subdoligranulum* showed a deviating trend and were enriched on both WB (1.70%) and WB280 (1.20%) when compared to the non-attached community (0.49%) (p=0.007, p=0.041 respectively). Within the ***Enterococcaceae*** family, *Enterococcus* species show the tendency to be enriched on WB280 compared to the control (p=0.05). A significant relative increase in ***Lactobacillaceae*** could be observed on the bran. Compared to a relative abundance of 0.37% of *Lactobacillaceae* in the non-attached community, abundances of 2% and 3% could be observed in the community associated with WB (p=0.009) and WB280 (p=0.003), respectively. This shift was solely caused by an increase in relative numbers of the genus *Lactobacillus* (Figure 3; Table S1, supplementary information).

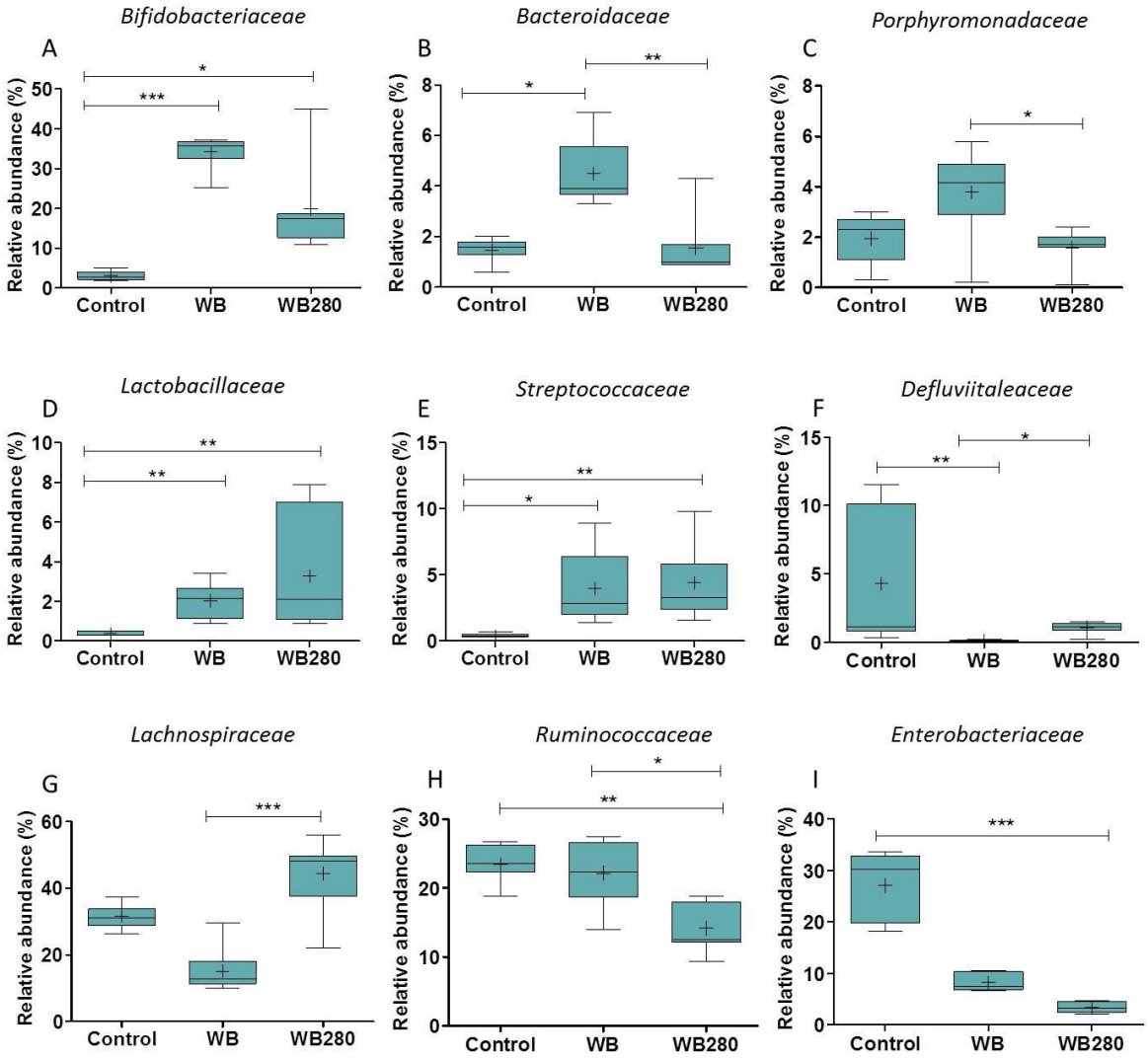


Figure 3 • Relative abundances of the most important bacterial families significantly differing between the non-attached and/or bran – associated communities. Bran fractions were fermented in a nutrient poor medium with a chicken cecal inoculum for 24 h at 37°C. Bran was omitted in the control fermentations. Non- and loosely attached bacteria were removed by washing prior to DNA extraction. Mean relative abundances are shown on the y-axis while the x-axis indicates the bran fraction or control for (A) *Bifidobacteriaceae*, (B) *Bacteroidaceae*, (C) *Porphyromonadaceae*, (D) *Lactobacillaceae*, (E) *Streptococcaceae*, (F) *Defluviitaleaceae*, (G) *Lachnospiraceae*, (H) *Ruminococcaceae*, and (I) *Enterobacteriaceae*, as determined by 16S rRNA V3-V4 amplicon sequencing. The plus represents the mean value, and the horizontal lines at the bottom, the middle and the top of the box represent the first quartile, median and third quartile, respectively. The whiskers indicate the min/max value. Significant differences between bran fractions and control are indicated with *$0.01<p<0.05$; **$0.001\leq p\leq 0.01$; ***$p<0.001$.

3.2 THE WHEAT BRAN-ATTACHED BACTERIAL COMMUNITY POTENTIALLY EXERTS DIFFERENT METABOLIC FUNCTIONS THAN THE NON-ATTACHED COMMUNITY

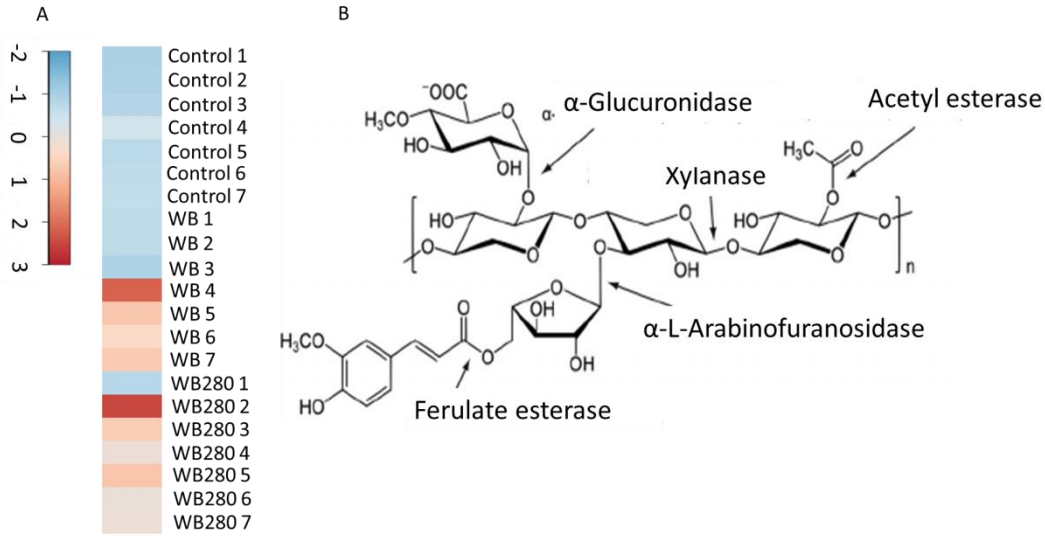


Figure 4 • Taxonomy-based functional profiling of bacterial communities attached and non-attached to WB and WB280 (A) and illustration of the activity of the enzymes involved in xylan degradation (B). The heatmap displays putative changes in taxonomy-based xylan degrading activity. Changes are displayed on a relative scale with enrichment in red and depletion in blue. Rightmost column indicates sample names. Heatmap was generated using the MetagenAssist software. Figure B was adapted from Rogowski et al. (2014).

Exploring the potential functions of the attached and non-attached communities reveals potentially distinct metabolic profiles. The communities attached to WB and WB280 were clearly enriched in OTUs assigned to xylan degraders (Figure 4A). Since one of the major constituents of wheat bran is arabinoxylan, the NCBI protein database was used to search for putative glycoside hydrolases and carbohydrate esterases which enable (partial) xylan degradation in taxa that were significantly enriched on bran material. Endoxylanases are responsible for the breakdown of the xylan backbone into smaller fragments. *Bacteroides thetaiotaomicron* encodes many enzymes, distributed over the different families: putative endoxylanases, xylosidases, arabinofuranosidases and glucuronidases can be traced back in the chosen reference genome. Also, *Bifidobacterium pseudolongum* features mostly arabinofuranosidases, which are accessory enzymes, assisting the xylosidases and endoxylanases (Figure 4B). In addition, it encodes a β-xylosidases. OTU5, showing 97% 16S rRNA sequence similarity with [*Ruminococcus] torques*, putatively expresses an endoxylanase and glucuronidase (Table S2, supplementary information)

3.3 INCLUSION OF WHEAT BRAN WITH DIFFERENT PARTICLE SIZES IN THE FEED OF BROILERS INDUCES PARTICLE SIZE DEPENDENT SHIFTS IN THE CECAL MICROBIOTA

One-day old chickens received a standard diet for ten consecutive days supplemented with WB or WB280. The control group received non-supplemented feed. Chickens were euthanized on day ten and cecal content was collected where after DNA was extracted and samples were sent for Illumina sequencing. A PCoA plot based on the Bray-Curtis dissimilarity index shows a specific clustering pattern of the cecal samples (Figure 5A). The cecal bacterial diversity of chickens from the control group differed significantly from that of chickens receiving WB and WB280. Also, the supplementation with either of two bran fractions had different effects on the bacterial community composition of the ceca ($p<0.001$). The richness, expressed as the number of observed OTUs, was significantly increased with 15% ($p=0.027$) for chickens receiving WB and 33% ($p<0.001$) for chickens receiving WB280 when compared with cecal contents from control chickens (Figure 5B).

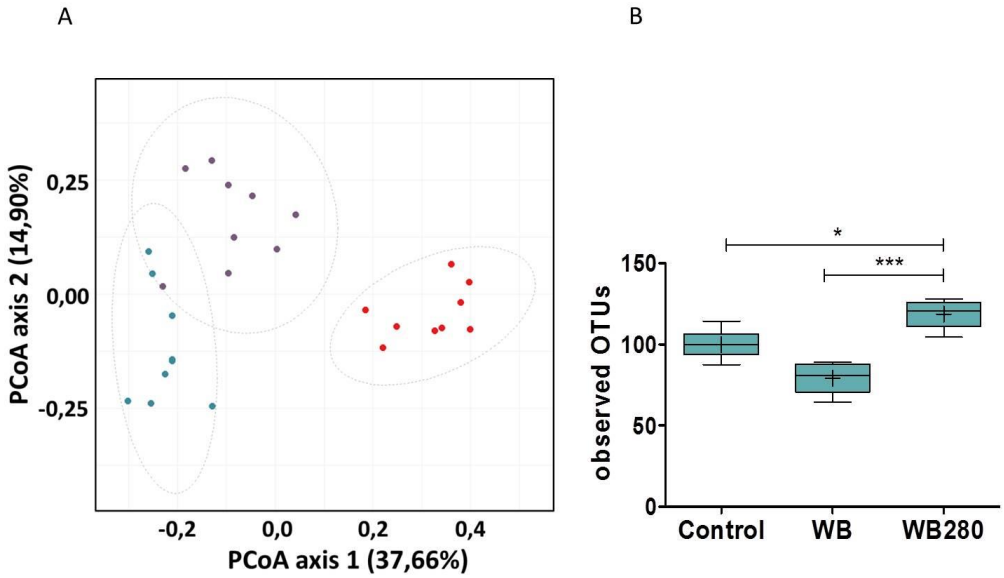


Figure 5 • Diversity and richness measures for the cecal bacterial communities of chickens in the control group, and the group receiving 1% WB and 1% WB280. One-day old chickens received a nonsupplemented standard diet (control ●; n=9), standard diet supplemented with 1% WB (●; n=8) or 1% WB280 (●; n=9) during 10 days. A PCoA biplot of the abundance based Bray-Curtis dissimilarity index revealed a distinctive cecal microbial community for chickens receiving a non-supplemented diet, 1% WB or 1% WB280 ($p<0.001$) (A). Richness of the different bacterial communities, expressed as number of observed OTUs (B). Significant differences between WB, WB280 and control are indicated with *$0.01<p<0.05$; ***$p<0.001$.

Relative abundances of members of the **Firmicutes** phylum were significantly higher in cecal contents of chickens receiving WB280 (94%) when compared to control group (82%,

p=0.008). A significant relative decrease in **Actinobacteria** could be observed for chickens fed WB (0.45%, p=0.002) and WB280 (1.95%, p=0.006) when compared to control chickens (12.5%) (Figure 6; Table S3, supplementary information).

At the family and genus level several significant shifts could be observed. The number of ***Enterobacteriaceae*** was significantly reduced in cecal contents from chickens receiving WB280 (0.67%) versus the control group (3.5%, p=0.021) and the group fed WB (8.4%, p<0.001) (Figure 7F). This shift at the family level could be fully explained by a decrease in relative numbers of the group *Escherichia*/*Shigella*. In accordance with the decrease of Actinobacteria in chickens fed wheat bran of either size, a decrease in ***Bifidobacteriaceae*** and members of the genus *Bifidobacterium* could be observed (Figure 7A). Overall numbers of ***Lachnospiraceae*** remained unchanged after the administration of wheat bran but specific genera within this family showed a significant shift. An enrichment of the genus *Pseudobutyrivibrio* could be observed in the cecal contents of chickens receiving WB when compared to control chickens (p=0.004) and chickens receiving WB280 (p=0.002). Members of the genus *Blautia* were increased in relative abundance in ceca of chickens receiving unsupplemented feed (14%, p=0.003) or feed with 1% WB280 (11%, p=0.03) compared to the group receiving WB (1%). A number of OTUs that could not be assigned to a specific genus within the *Lachnospiraceae* or that was not cultivable, was enriched in cecal contents from chickens receiving bran when compared to control chickens. This was significant different for the group receiving WB (p=0.015). The intake of WB280 appeared to increase numbers of ***Ruminococcaceae***

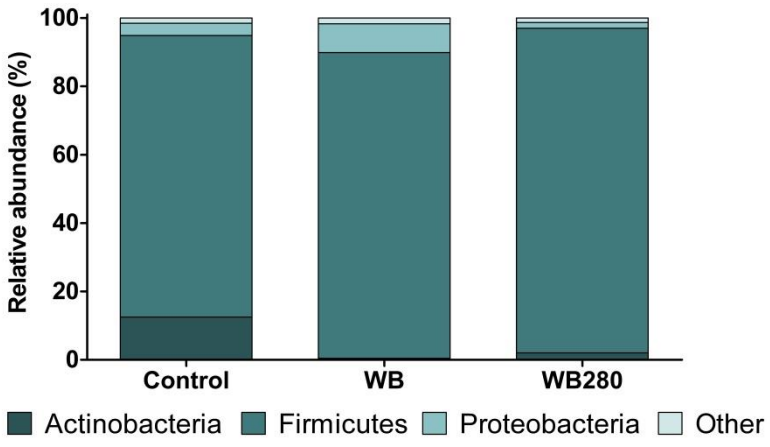


Figure 6 • Relative abundances of the most important bacterial phyla present in cecal contents of broilers receiving bran with different particle size. A standard broiler feed was supplemented with either 1% WB or WB280 during ten days. DNA was extracted from cecal contents and sent for 16S rRNA V3-V4 amplicon sequencing. Cecal community composition was compared between chickens from the control group receiving a standard feed and groups of chickens receiving WB or WB280.

compared to the intake of regular wheat bran and the control-fed chickens (p=0.018, p=0.032 respectively) (Figure 7E). Within the *Ruminococcaceae*, the genus *Anaerotruncus* was represented less in the ceca of chickens receiving WB than in the ceca of control chickens (p=0.005) and the group of chickens receiving WB280 (p<0.001). Members of the genus *Subdoligranulum* were specifically enriched in cecal contents of chickens receiving WB280 compared to the group receiving non-supplemented feed (p=0.015) and WB (p=0.012). When summing the relative abundance of *Lachnospiraceae* and *Ruminococcaceae*, a specific trend could be observed: chickens fed WB280 showed higher relative numbers in their cecal contents compared to control chickens (73% vs 54%, p=0.024) (Figure 7G). In accordance, the number of gene copies encoding the butyryl-CoA:acetate-CoA transferase, as determined with qPCR, was significantly higher in the cecal contents of chickens receiving WB280 than in those of control chickens (p=0.02), while total numbers of bacteria, determined by quantifying total bacterial 16S rRNA gene copies, remained unchanged (Figure 8).

.

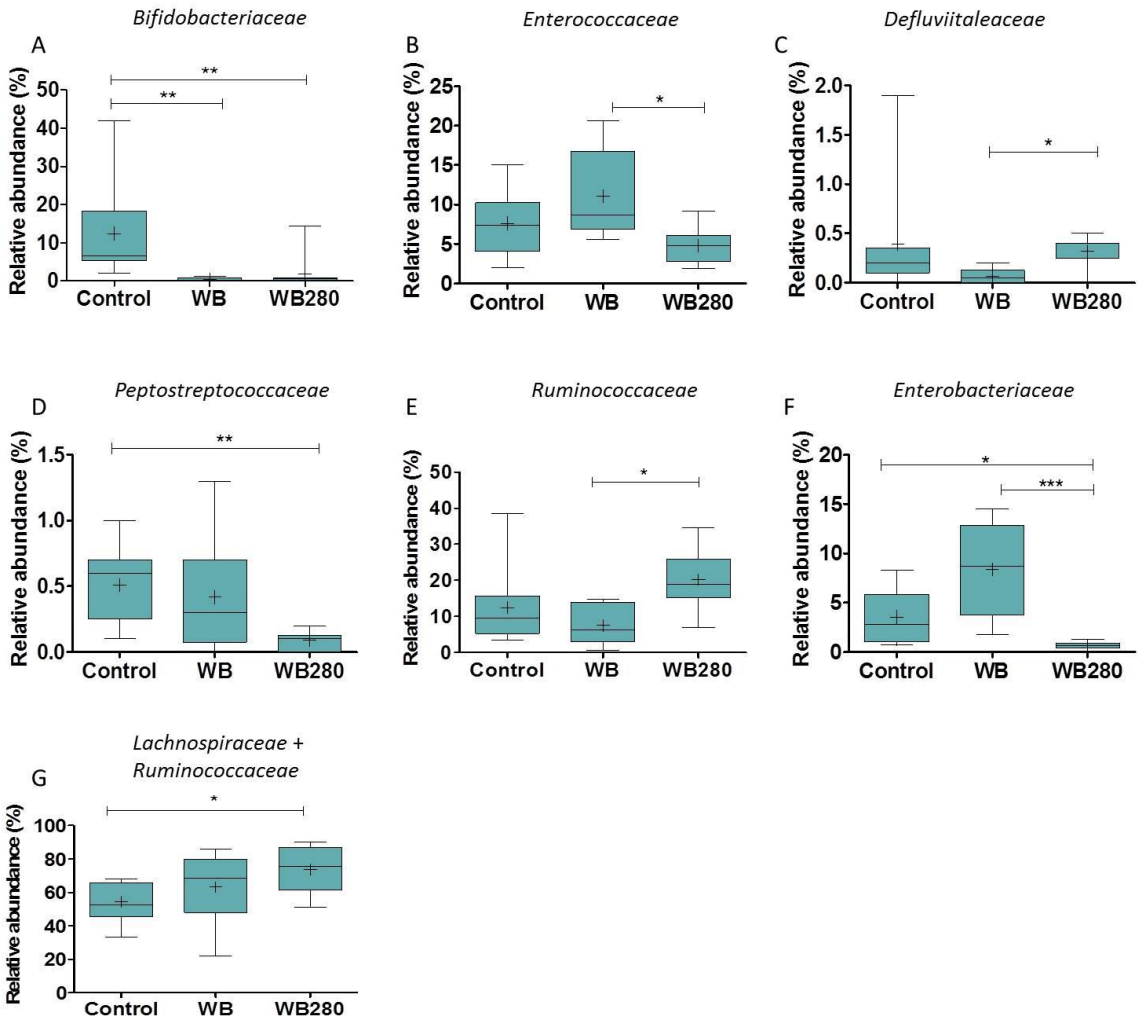


Figure 7 • Relative abundance of the most important bacterial families significantly differing between groups. Chickens were fed a standard commercial feed during 10 days. Group 1 received an unsupplemented feed (control), while group 2 and 3 were fed the standard feed supplemented with 1% WB and 1% WB280, respectively. On day 10 chickens were euthanized and DNA was extracted from the cecal contents. Mean relative abundances are shown on the y-axis while the x-axis indicates the treatment for (A) *Bifidobacteriaceae*, (B) *Enterococcaceae*, (C) Defluviitaleaceae, (D) Peptostreptococcaceae, (E) *Ruminococcaceae*, (F*) Enterobacteriaceae* and (G) the sum of *Lachnospiraceae* and *Ruminicoccaceae*, as determined by Illumina sequencing. The plus represents the mean value and the horizontal lines at the bottom, the middle and the top of the box represent the first quartile, median and third quartile, respectively. The whiskers indicate the min/max value. Significant differences between bran fractions and control are indicated with *$0.01<p<0.05$; **$0.001\leq p\leq 0.01$; ***$p<0.001$.

4. DISCUSSION

Previous human microbiota studies indicated that wheat bran is colonized by a specific bacterial community (Leitch et al. 2007, Walker et al. 2008, De Paepe et al. 2017). In the current study we investigated whether reducing the particle size of wheat bran could alter and/or optimize the colonizing community using a cecal inoculum from broilers. We also investigated the effect of administration of WB and WB280 on the cecal community composition of broilers *in vivo* to evaluate its potential to induce a microbial community shift which is deemed to be beneficial.

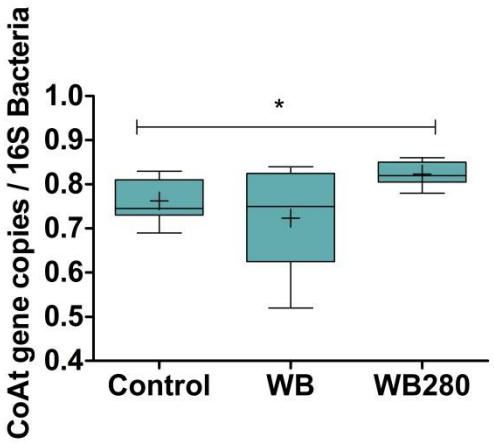


Figure 8 • Relative number of *CoAt* gene copies. The y-axis shows the number of *CoAt* gene copies relatively to the number of total bacterial 16S rRNA gene copies of ten-day old broilers fed a standard feed (control), or a standard feed supplemented with either 1% WB or 1% WB280. The plus represents the mean value and the horizontal lines at the bottom, the middle and the top of the box represent the first quartile, median and third quartile, respectively. The whiskers indicate the min/max value Significant differences between bran fractions and control are indicated with *0.01<p<0.05.

Clear compositional differences were detected in the wheat bran-attached and non-attached communities. These results are in accordance with those of Walker et al. (2008), who previously stated that the species composition of the human intestinal microbiota clearly differs between particle-associated and liquid phase communities (Walker et al. 2008). De Paepe et al. (2017) confirmed this by characterizing wheat bran attached and non-attached bacterial communities, using 16S rRNA amplicon sequencing. These findings contrast with the observations made by Macfarlane & Macfarlane (2006). They could not find significant differences between the biofilm community and a non-adherent population. However, they observed significant differences in fermentation patterns between strongly adherent and non-adherent bacteria: the fermentation of small, highly soluble oligosaccharides was invariably more rapidly executed by non-adherent bacteria (Macfarlane & Macfarlane 2006).

Species colonizing the surface of dietary particles are likely to encode a specific enzymatic armory that enables both attachment and degradation of complex insoluble polysaccharides (Lombard et al. 2014). In comparison to the non-attached community, the wheat bran-attached

community may be metabolically adapted to degrade xylan. The community attached to wheat bran was enriched in OTUs which were taxonomically assigned to xylan degrading species such as *Bacteroides thetaiotaomicron*, *Bifidobacterium pseudolongum* and *[Ruminococcus] torques*, while the non-attached community seemed to be depleted in OTUs linked to taxa known for exerting this metabolic function. *Bacteroides thetaiotaomicron* possesses notorious carbohydrate degradation activities, encoding glycoside hydrolases (GHs) belonging to as many as 56 different families (Xu et al. 2003). Many members of the Bacteroidetes phylum are equipped with an extended armory of carbohydrate active enzymes (CAZymes) (Xu et al. 2003, Flint et al. 2008, Flint et al. 2012, El Kaoutari et al. 2013), so it is likely that the Bacteroidetes strain present in the wheat bran – attached community also has a high enzymatic potential. The carbohydrate catabolism of Firmicutes may not be underestimated either. *Roseburia intestinalis* for example, is reported for extensive xylan-utilization (Duncan et al. 2002). In addition to Bacteroidetes and Firmicutes species, Kaoutari et al. (2013) have shown that *Bifidobacterium* species, such as *B. longum*, are also involved in carbohydrate metabolism by encoding several putative GHs (El Kaoutari et al. 2013). Our results show an enrichment of an OTU assigned to *B. pseudolongum* on both WB and WB280, which encodes, according to the NCBI database, xylosidases and arabinofuranosidases. These enzymes enable both the breakdown of the xylan backbone, as well as release of the most important substituents (Waeonukul 2013, Rogowski et al. 2014).

It was demonstrated before that the community composition of insoluble substrates strongly depends on the nature of the substrate (Leitch et al. 2007). In a fermentation experiment, mucus, wheat bran or starch were colonized by very distinct bacterial communities (Leitch et al. 2007, Walker et al. 2008). In the present study, we showed that also the size of particles has an influence on the community composition. By reducing the particle size, one does not alter the chemical composition of the bran material but the availability of some of its constituents is increased (Jacobs et al. 2016). A distinctive colonization pattern for WB and the technically modified WB280 fraction could be observed: both fractions were enriched with lactate producing genera such as *Lactobacillus* and *Bifidobacterium* but, compared to WB, a significant enrichment of *Lachnospiraceae* on WB280 could be observed. In addition to a large number of OTUs which could not be appointed to an established genus within the *Lachnospiraceae*, also the genus *Roseburia* was responsible for this shift. Several members of

this *Lachnospiraceae* family have the ability to consume lactate and produce butyrate (Belenguer et al. 2006, De Maesschalck et al. 2015a). Cross-feeding, and thus butyrate production, could be enhanced between these metabolic groups through their co-occurrence on WB280. It may be questioned whether the transit rate in the chicken intestine is sufficiently low to enable the formation of particle associated communities. Probably the longer retention time in the cecal pouches poses an answer to this challenge (Clench & Mathias 1992, Clench 1999). Because they are the main site for fermentation and because they are characterized by a higher bacterial richness and diversity than any other part of the chicken GIT, the ceca and its inhabitants have a strong impact on the uptake and utilization of energy and nutrients (Choct et al. 1996, Lan et al. 2005, Shaufi et al. 2015). The cecal bacterial community can reach up to 10^{12} cells/g of content and is dominated by three bacterial phyla accounting for more than 90% of all intestinal bacteria: Firmicutes (dominant), Bacteroidetes and Proteobacteria, each consisting of numerous families, genera and species (Apajalahti et al. 2004, Wei et al. 2013, Oakley & Kogut 2016).

The specific reluctance of members of the *Enterobacteriaceae* family to attach to small particle size bran may be explained by the presence of this butyrogenic consortium. It is known that SCFA such as butyrate and propionate have inhibitory effects on *Salmonella*, a zoonotic agent belonging to the *Enterobacteriaceae* (Van Immerseel et al. 2003, Van Immerseel et al. 2004, Van Immerseel et al. 2006, Van Immerseel et al. 2009, Vermeulen et al. 2017). These effects seem to radiate from the bran material to the entire cecal content since relatively less *Enterobacteriaceae* were present in cecal contents of WB280-fed broilers compared to control-fed chickens. This might be a consequence of the co-occurring increase in relative numbers of butyrate producing bacteria (sum of *Lachnospiraceae* and *Ruminococcaceae*). We have previously shown that the addition of 1% WB280 to the feed of broilers could reduce the cecal colonization of *Salmonella*, whereas this effect could not be achieved when administering WB (Vermeulen et al. 2017). It was hypothesized that through particle size reduction, the bran constituents become more available for fermentation by intestinal bacteria due to breaking of cell wall barriers and/or an increased accessible surface (Jacobs et al. 2015). In addition, the presence of a specifically attached butyrogenic community could be responsible for the more efficient fermentation of WB280 and increased production of SCFA.

We can conclude that the technical modification of wheat bran, here being particle size reduction, induces the colonization of a very specific butyrogenic community compared to non-modified wheat bran. Bacteria with the appropriate enzyme arsenal seem to be attracted to the bran fractions. When WB280 is administered to broilers, shifts in the total cecal community can be induced, similar to the shifts observed in the particle-associated community *in vitro*. The intake of fibre rich diets has been associated with increased Firmicutes and decreased Proteobacteria numbers (Simpson & Campbell 2015). Here we show that applying only 1% of particle size reduced wheat bran can ameliorate the ratio of Firmicutes to Proteobacteria. This could imply that in future intervention studies one should take the particle size of DF into account.

ACKNOWLEDGMENTS

The authors would like to thank the “Vlaams Agentschap Innoveren en Ondernemen” (Vlaio, former IWT or “Agentschap voor Innovatie door Wetenschap en Technologie ”) for the financial support of the SBO BRANDING project (grant no. 130028).

COMPETING INTERESTS

K. Vermeulen, J. Verspreet, C.M. Courtin, F. Van Immerseel and R. Ducatelle are listed as coinventors on a patent application for a wheat bran fraction for use to control *Salmonella* infection (International Application Number PCT/EP2017/067028).

SUPPLEMENTARY INFORMATION

Table S1

Relative abundances of bacterial families and genera in the non-attached community and the community attached to WB and WB280, as determined by 16S rRNA V3-V4 amplicon sequencing.

Phylum	Family	Genus	Control Mean ± SD	WB Mean ± SD	WB280 Mean ± SD
Firmicutes			61.03 ± 5.11^{ab}	45.88 ± 3.56^{a}	70.63 ± 14.59^{b}
	Enterococcaceae				
		Enterococcus	0.40 ± 0.55^{a*}	2.03 ± 1.37^{ab}	3.17 ±4.12^{b}*
	Lactobacillaceae		0.37 ± 0.10^{a}	2.03 ± 0.90^{b}	3.29 ± 2.93^{b}
		Lactobacillus	0.84 ± 1.31^{a}	2.00 ± 0.84^{ab}	3.29 ± 2.93^{b}
	Defluviitaleaceae		4.29 ± 4.77^{a}	0.07 ± 0.08^{b}	1.04 ± 0.42^{a}
	Lachnospiraceae		31.66 ± 3.55^{ab}	15.13 ± 7.19^{a}	44.40 ± 11.24^{b}
		Coprococcus	0.23 ± 0.14^{ab}	0.10 ± 0.09^{a}	1.10 ± 0.93^{b}
		Roseburia	0.01± 0.04^{a}	0.03± 0.05^{ab}	0.11 ± 0.09^{b}
		Lachnospiraceae, other/uncultured	28.67 ± 2.76^{ab}	12.17 ± 7.55^{a}	41.07 ± 10.57^{b}
	Ruminococcaceae		23.47 ± 2.63^{a}	22.17 ± 4.83^{a}	14.16 ± 3.48^{b}
		Anaerotruncus	4.73 ± 1.39^{a}	1.82 ± 0.31^{ab}	1.16 ± 0.28^{b}
		Subdoligranulum	0.49 ± 0.20^{a}	1.73 ± 1.03^{b}	1.23 ± 0.57^{b}
		Ruminococcaceae, other/uncultured	13.01 ± 2.33^{a}	8.95 ± 0.92^{ab}	6.60 ± 1.85^{b}
	Streptococcaceae		0.39 ± 0.21^{a}	3.97 ± 2.80^{b}	4.41 ± 2.83^{b}
		Streptococcus	0.67 ± 0.60^{a}	3.97 ±2.80^{b}	4.41 ± 2.83^{b}
Actinobacteria			3.77 ± 0.95^{a}	34.28 ± 4.47^{b}	19.93 ± 11.39^{b}
	Bifidobacteriaceae		3.17 ± 1.10^{a}	34.23 ± 4.50^{b}	19.90 ± 11.41^{b}
		Bifidobacterium	5.56 ± 6.76^{a}	34.23 ± 4.50^{b}	19.90 ± 11.41^{ab}
	Eggerthellaceae		0.60 ± 0.36^{a}	0.05 ± 0.05^{b}	0.04 ± 0.05^{b}
		Eggerthella	0.21 ± 0.12^{a}	0.03 ± 0.05^{b}	0.00 ± 0.00^{b}
		Gordonibacter	0.36 ± 0.27^{a}	0.02 ± 0.04^{b}	0.01 ± 0.04^{b}

Bacteroidetes			3.09 ± 0.98[b]	8.33 ± 0.99[a]	3.21 ± 0.86[b]
	Bacteroidaceae		1.47 ± 0.46[a]	4.48 ± 1.33[b]	1.56 ± 1.24[a]
		Bacteroides	1.66 ± 0.62[a]	4.48 ± 1.33[b]	1.56 ± 1.24[a]
	Porphyromonadaceae		1.94 ± 0.98[ab]	3.78 ± 1.89[a]	1.59 ± 0.72[b]
		Parabacteroides	2.17 ± 0.67[ab]	3.78 ±1.89[a]	1.59 ± 0.72[b]
Proteobacteria			29.60 ± 4.73[a]	9.45 ± 1.53[ab]	4.00 ± 0.87[b]
	Enterobacteriaceae		27.06 ± 6.24[a]	8.22 ± 1.74[ab]	3.43 ± 1.00[b]
		Escherichia/Shigella	22.80 ± 7.02[a]	8.05 ± 1.55[ab]	3.37 ± 0.98[b]

Mean relative abundance ± standard deviation (SD) are shown.
[ab] Significant differences ($p<0.05$) between bran fractions and control are indicated with different letters and highlighted in boldface.
[*] $p = 0.05$

Table S2

Presence of enzymes involved in xylan degradation in species specifically enriched on bran material. The presence of specific xylan degradation enzymes was explored using the NCBI protein databases. The search was restricted to OTUs significantly enriched on bran material. The OTU and corresponding BLAST hit are shown with accession number. For each BLAST hit a reference genome was chosen and searched for putative xylan degradation enzymes.

				Treatment			Xylan degrading enzymes					
	ID (% 16S rRNA sequence similarity	Accession number	Representative genome	Control (Mean ± SD)	WB (Mean ± SD)	WB280 (Mean ± SD)	β xylosidase	Endoxylanase	Arabinofuranosidase	Glucuronidase	Acetyl xylan esterase	Feruloyl esterase
OTU2	99% *Bifidobacterium pseudolongum*	LC187663.1	NZ_CP007457.1	0.60 ±0.42[a]	27.62 ± 3.17[b]	16.87 ± 12.22[b]	AIZ15701.1; AIZ15702.1;		AIZ15573.1; AIZ15705.1; AIZ15987.1; AIZ15995.1;			
OTU15	100% *Bacteroides thetaiotaomicron*	KU234409.1	NC_004663.1	1.48 ± 0.43[a]	4.47 ± 1.31[b]	1.55 ± 1.23[a]	NP_809057.1; NP_809058.1; NP_811764.1; NP_810694.1; NP_812006.1; NP_812020.1; NP_812567.1; NP_812574.1; NP_813006.1; NP_813096.1; NP_813625.1	NP_809282.1; NP_809921.1; NP_810000.1; NP_810105.1; NP_811807.1; NP_811810.1; NP_811955.1; NP_812573.1; NP_812586.1; NP_812988.1	NP_809261.1; NP_809281.1; NP_812008.1; NP_812568.1	NP_811535.1; NP_812204.1; NP_813062.1	NP_813091.1;	
OTU5	97% *[Ruminococcus] torques*	AB910746.1	NZ_AAVP00000000.2	3.57 ± 0.24[a]	3.52 ± 1.73[a]	15.39 ± 3.71[b]		CBL27152.1		CBL26599.1		

Mean relative abundance ± standard deviation (SD) are shown.

[ab] Significant differences ($p<0.05$) between bran fractions and control are indicated with different letters.

Table S3

Relative abundances of bacterial families and genera in the microbial community of the ceca of chickens receiving wheat bran with different particle size, as determined by 16S rRNA V3-V4 amplicon sequencing.

Phylum	Family	Genus	Control Mean ± SD	WB Mean ± SD	WB280 Mean ± SD
Firmicutes			82.32 ±11.63[a]	89.35 ± 4.78[ab]	93.87 ± 6.89[b]
	Enterococcaceae		7.61 ± 4.14[ab]	11.05 ± 5.81[a]	4.77 ± 2.34[b]
		Enterococcus	7.61 ± 4.14[ab]	11.05 ± 5.81[a]	4.77 ± 2.34[b]
	Defluviitaleaceae		0.39 ± 0.58[ab]	0.07 ± 0.08[a]	0.32 ± 0.15[b]
	Lachnospiraceae				
		Blautia	13.83 ± 7.14[a]	1.23 ± 1.47[b]	11.11 ± 6.27[a]
		Pseudobutyrivibrio	0.27 ± 0.37[a]	1.98 ± 1.74[b]	0.08 ± 0.14[a]
		Lachnospiraceae, other/uncultured	30.38 ± 8.91[a]	51.41 ± 18.27[b]	42.31 ± 12.17[ab]
	Peptostreptococcaceae		0.51 ± 0.28[a]	0.42 ± 0.47[ab]	0.09 ± 0.07[b]
	Ruminococcaceae		12.42 ± 10.70[a]	7.58 ± 5.56[a]	20.22 ± 8.24[b]
		Anaerotruncus	1.24 ± 0.44[a]	0.06 ± 0.05[b]	1.71 ± 0.92[a]
		Subdoligranulum	0.83 ± 1.32[a]	1.05 ± 1.59[a]	7.17 ± 4.65[b]
Actinobacteria			12.50 ± 12.46[a]	0.45 ± 0.45[b]	1.95 ± 4.35[b]
	Bifidobacteriaceae		12.44 ± 12.49[a]	0.45 ± 0.45[b]	1.86 ± 4.38[b]
		Bifidobacterium	12.44 ± 12.97[a]	0.45 ± 0.45[b]	1.94 ± 4.64[b]
Proteobacteria					
	Enterobacteriaceae		3.54 ± 2.74[a]	8.40 ± 4.97[a]	0.67 ± 0.11[b]
		Escherichia/Shigella	3.32 ± 2.56[a]	6.20 ± 3.13[a]	0.77 ± 1.01[b]

Mean ± SD are shown.

[ab] Significant differences ($p<0.05$) between bran fractions and control are indicated with different letters and highlighted in boldface.

REFERENCES

Apajalahti, J., Kettunen, A. and Graham, H. Characteristics of the Gastrointestinal Microbial Communities, with Special Reference to the Chicken. (2004) *Worlds Poultry Science Journal* **60**, 2: 223-232.

Arndt, D., Xia, J., Liu, Y., Zhou, Y., Guo, A.C., Cruz, J.A., Sinelnikov, I., Budwill, K., Nesbø, C.L. and Wishart, D.S. Metagenassist: A Comprehensive Web Server for Comparative Metagenomics. (2012) *Nucleic Acids Research* **40**, W1: W88-W95.

Belenguer, A., Duncan, S.H., Calder, A.G., Holtrop, G., Louis, P., Lobley, G.E. and Flint, H.J. Two Routes of Metabolic Cross-Feeding between *Bifidobacterium Adolescentis* and Butyrate-Producing Anaerobes from the Human Gut. (2006) *Applied and Environmental Microbiology* **72**, 5: 3593-3599.

Biggs, P. and Parsons, C. The Effect of Oligosaccharides on Growth Performance, Nutrient Utilization. And Cecal Microbes in Young Chicks. (2005) *Poultry Science* **84**: 69-69.

Caporaso, J.G., Bittinger, K., Bushman, F.D., DeSantis, T.Z., Andersen, G.L. and Knight, R. Pynast: A Flexible Tool for Aligning Sequences to a Template Alignment. (2010a) *Bioinformatics* **26**, 2: 266-7.

Caporaso, J.G., Kuczynski, J., Stombaugh, J., Bittinger, K., Bushman, F.D., Costello, E.K., Fierer, N., Pena, A.G., Goodrich, J.K., Gordon, J.I., Huttley, G.A., Kelley, S.T., Knights, D., Koenig, J.E., Ley, R.E., Lozupone, C.A., McDonald, D., Muegge, B.D., Pirrung, M., Reeder, J., Sevinsky, J.R., Turnbaugh, P.J., Walters, W.A., Widmann, J., Yatsunenko, T., Zaneveld, J. and Knight, R. Qiime Allows Analysis of High-Throughput Community Sequencing Data. (2010b) *Nature Methods* **7**, 5: 335-6.

Choct, M., Hughes, R.J., Wang, J., Bedford, M.R., Morgan, A.J. and Annison, G. Increased Small Intestinal Fermentation Is Partly Responsible for the Anti-Nutritive Activity of Non-Starch Polysaccharides in Chickens. (1996) *British Poultry Science* **37**, 3: 609-21.

Clench, M.H. The Avian Cecum: Update and Motility Review. (1999) *Journal of Experimental Zoology* **283**, 4-5: 441-447.

Clench, M.H. and Mathias, J.R. Intestinal Transit - How Can It Be Delayed Long Enough for Birds to Act as Long-Distance Disperal Agents. (1992) *The Auk* **109**, 4: 933-936.

Codex Alimentarius Commission. "Report of the 31th Session of the Codex Committee on Nutrition and Foods for Special Dietary Uses." In *Codex Alimentarius Commission*. Rome, **2009**.

Courtin, C.M., Swennen, K., Broekaert, W.F., Swennen, Q., Buyse, J., Decuypere, E., Michiels, C.W., Ketelaere, B.D. and Delcour, J.A. Effects of Dietary Inclusion of Xylooligosaccharides, Arabinoxylooligoscaccharides and Soluble Arabinoxylan on the Microbial Composition of Caecal Contents of Chickens. (2008) *Journal of the Science of Food and Agriculture* **88**, 14: 2517-2522.

Crittenden, R., Karppinen, S., Ojanen, S., Tenkanen, M., Fagerstrom, R., Matto, J., Saarela, M., Mattila-Sandholm, T. and Poutanen, K. In Vitro Fermentation of Cereal Dietary Fibre Carbohydrates by Probiotic and Intestinal Bacteria. (2002) *Journal of the Science of Food and Agriculture* **82**, 8: 781-789.

Damen, B., Verspreet, J., Pollet, A., Broekaert, W.F., Delcour, J.A. and Courtin, C.M. Prebiotic Effects and Intestinal Fermentation of Cereal Arabinoxylans and Arabinoxylan Oligosaccharides in Rats Depend Strongly on Their Structural Properties and Joint Presence. (2011) *Molecular Nutrition & Food Research* **55**, 12: 1862-1874.

De Maesschalck, C., Eeckhaut, V., Maertens, L., De Lange, L., Marchal, L., Nezer, C., De Baere, S., Croubels, S., Daube, G., Dewulf, J., Haesebrouck, F., Ducatelle, R., Taminau, B. and Van Immerseel, F. Effects of Xylo-Oligosaccharides on Broiler Chicken Performance and Microbiota. (2015a) *Applied and Environmental Microbiology* **81**, 17: 5880-5888.

De Maesschalck, C., Eeckhaut, V., Maertens, L., De Lange, L., Marchal, L., Nezer, C., De Baere, S., Croubels, S., Daube, G., Dewulf, J., Haesebrouck, F., Ducatelle, R., Taminau, B. and Van

Immerseel, F. Effects of Xylo-Oligosaccharides on Broiler Chicken Performance and Microbiota. (2015b) *Applied Environmental Microbiology* **81**, 17: 5880-5888.

De Paepe, K., Kerckhof, F.M., Verspreet, J., Courtin, C.M. and Van de Wiele, T. Inter-Individual Differences Determine the Outcome of Wheat Bran Colonization by the Human Gut Microbiome. (2017) *Environmental Microbiology*.

Duncan, S.H., Hold, G.L., Barcenilla, A., Stewart, C.S. and Flint, H.J. *Roseburia Intestinalis* Sp Nov., a Novel Saccharolytic, Butyrate-Producing Bacterium from Human Faeces. (2002) *International Journal of Systematic and Evolutionary Microbiology* **52**: 1615-1620.

Edgar, R.C. Uparse: Highly Accurate Otu Sequences from Microbial Amplicon Reads. (2013) *Nature Methods* **10**, 10: 996-8.

Edgar, R.C., Haas, B.J., Clemente, J.C., Quince, C. and Knight, R. Uchime Improves Sensitivity and Speed of Chimera Detection. (2011) *Bioinformatics* **27**, 16: 2194-200.

El Kaoutari, A., Armougom, F., Gordon, J.I., Raoult, D. and Henrissat, B. The Abundance and Variety of Carbohydrate-Active Enzymes in the Human Gut Microbiota. (2013) *Nature Reviews Microbiology* **11**, 7: 497-504.

Flint, H.J., Bayer, E.A., Rincon, M.T., Lamed, R. and White, B.A. Polysaccharide Utilization by Gut Bacteria: Potential for New Insights from Genomic Analysis. (2008) *Nature Reviews Microbiology* **6**, 2: 121-131.

Flint, H.J., Scott, K.P., Duncan, S.H., Louis, P. and Forano, E. Microbial Degradation of Complex Carbohydrates in the Gut. (2012) *Gut Microbes* **3**, 4: 289-306.

Geier, M.S., Torok, V.A., Allison, G.E., Ophel-Keller, K. and Hughes, R.J. Indigestible Carbohydrates Alter the Intestinal Microbiota but Do Not Influence the Performance of Broiler Chickens. (2009) *Journal of Applied Microbiology* **106**, 5: 1540-1548.

Griffiths, R.I., Whiteley, A.S., O'Donnell, A.G. and Bailey, M.J. Rapid Method for Coextraction of DNA and Rna from Natural Environments for Analysis of Ribosomal DNA- and Rrna-Based Microbial Community Composition. (2000) *Applied and Environmental Microbiology* **66**, 12: 5488-5491.

Hemdane, S., Jacobs, P.J., Dornez, E., Verspreet, J., Delcour, J.A. and Courtin, C.M. Wheat (Triticum Aestivum L.) Bran in Bread Making: A Critical Review. (2016) *Comprehensive Reviews in Food Science and Food Safety* **15**, 1: 28-42.

Hopkins, M.J., Macfarlane, G.T., Furrie, E., Fite, A. and Macfarlane, S. Characterisation of Intestinal Bacteria in Infant Stools Using Real-Time Pcr and Northern Hybridisation Analyses. (2005) *Fems Microbiology Ecology* **54**, 1: 77-85.

Howlett, J.F., Betteridge, V.A., Champ, M., Craig, S.A.S., Meheust, A. and Jones, J.M. The Definition of Dietary Fiber – Discussions at the Ninth Vahouny Fiber Symposium: Building Scientific Agreement. (2010) *Food & Nutrition Research* **54**: 10.3402/fnr.v54i0.5750.

Ihrmark, K., Bodeker, I.T., Cruz-Martinez, K., Friberg, H., Kubartova, A., Schenck, J., Strid, Y., Stenlid, J., Brandstrom-Durling, M., Clemmensen, K.E. and Lindahl, B.D. New Primers to Amplify the Fungal Its2 Region--Evaluation by 454-Sequencing of Artificial and Natural Communities. (2012) *FEMS Microbiology Ecology* **82**, 3: 666-77.

Jacobs, P.J., Bogaerts, S., Hemdane, S., Delcour, J.A. and Courtin, C.M. Impact of Wheat Bran Hydration Properties as Affected by Toasting and Degree of Milling on Optimal Dough Development in Bread Making. (2016) *Journal of Agricultural and Food Chemistry* **64**, 18: 3636-3644.

Jacobs, P.J., Hemdane, S., Dornez, E., Delcour, J.A. and Courtin, C.M. Study of Hydration Properties of Wheat Bran as a Function of Particle Size. (2015) *Food Chemistry* **179**: 296-304.

Johnson, I.T. New Food Components and Gastrointestinal Health. (2001) *Proceedings of the Nutrition Society* **60**, 4: 481-8.

Kowalchuk, G.A., Naoumenko, Z.S., Derikx, P.J.L., Felske, A., Stephen, J.R. and Arkhipchenko, I.A. Molecular Analysis of Ammonia-Oxidizing Bacteria of the Beta Subdivision of the Class Proteobacteria in Compost and Composted Materials. (1999) *Applied and Environmental Microbiology* **65**, 2: 396-403.

Kumar, V., Sinha, A.K., Makkar, H.P., de Boeck, G. and Becker, K. Dietary Roles of Non-Starch Polysaccharides in Human Nutrition: A Review. (2012) *Critical Reviews in Food Science and Nutrition* **52**, 10: 899-935.

Lan, Y., Verstegen, M.W.A., Tamminga, S. and Williams, B.A. The Role of the Commensal Gut Microbial Community in Broiler Chickens. (2005) *Worlds Poultry Science Journal* **61**, 1: 95-104.

Larue, R., Yu, Z., Parisi, V.A., Egan, A.R. and Morrison, M. Novel Microbial Diversity Adherent to Plant Biomass in the Herbivore Gastrointestinal Tract, as Revealed by Ribosomal Intergenic Spacer Analysis and Rrs Gene Sequencing. (2005) *Environmental Microbiology* **7**, 4: 530-43.

Leitch, E.C.M., Walker, A.W., Duncan, S.H., Holtrop, G. and Flint, H.J. Selective Colonization of Insoluble Substrates by Human Faecal Bacteria. (2007) *Environmental Microbiology* **9**, 3: 667-679.

Lombard, V., Ramulu, H.G., Drula, E., Coutinho, P.M. and Henrissat, B. The Carbohydrate-Active Enzymes Database (Cazy) in 2013. (2014) *Nucleic Acids Research* **42**, D1: D490-D495.

Louis, P. and Flint, H.J. Development of a Semiquantitative Degenerate Real-Time Pcr-Based Assay for Estimation of Numbers of Butyryl-Coenzyme a (Coa) Coa Transferase Genes in Complex Bacterial Samples. (2007) *Applied and Environmental Microbiology* **73**, 6: 2009-2012.

Macfarlane, S. and Macfarlane, G.T. Composition and Metabolic Activities of Bacterial Biofilms Colonizing Food Residues in the Human Gut. (2006) *Applied and Environmental Microbiology* **72**, 9: 6204-6211.

Maes, C. and Delcour, J.A. Alkaline Hydrogen Peroxide Extraction of Wheat Bran Non-Starch Polysaccharides. (2001) *Journal of Cereal Science* **34**, 1: 29-35.

Maes, C. and Delcour, J.A. Structural Characterisation of Water-Extractable and Water-Unextractable Arabinoxylans in Wheat Bran. (2002) *Journal of Cereal Science* **35**, 3: 315-326.

McDonald, D., Clemente, J.C., Kuczynski, J., Rideout, J.R., Stombaugh, J., Wendel, D., Wilke, A., Huse, S., Hufnagle, J., Meyer, F., Knight, R. and Caporaso, J.G. The Biological Observation Matrix (Biom) Format Or: How I Learned to Stop Worrying and Love the Ome-Ome. (2012) *Gigascience* **1**, 1: 7.

Michalet-Doreau, B., Fernandez, I., Peyron, C., Millet, L. and Fonty, G. Fibrolytic Activities and Cellulolytic Bacterial Community Structure in the Solid and Liquid Phases of Rumen Contents. (2001) *Reproduction, Nutrition, Development* **41**, 2: 187-94.

Moura, P., Barata, R., Carvalheiro, F., Girio, F., Loureiro-Dias, M.C. and Esteves, M.P. *In Vitro* Fermentation of Xylo-Oligosaccharides from Corn Cobs Autohydrolysis by *Bifidobacterium* and *Lactobacillus* Strains. (2007) *Food Science and Technology* **40**, 6: 963-972.

Oakley, B.B. and Kogut, M.H. Spatial and Temporal Changes in the Broiler Chicken Cecal and Fecal Microbiomes and Correlations of Bacterial Taxa with Cytokine Gene Expression. (2016) *Frontiers in Veterinary Science* **3**: 11.

Oksanen J, B.G., Kindt R, Legendre P, Minchin P R, O'Hara R B, Gavin L, Simpson P, Solymos M, Henry H, Stevens H W. Vegan: Community Ecology Package. R Package Version 2 0-10. (2010).

Quast, C., Pruesse, E., Yilmaz, P., Gerken, J., Schweer, T., Yarza, P., Peplies, J. and Glockner, F.O. The Silva Ribosomal Rna Gene Database Project: Improved Data Processing and Web-Based Tools. (2013) *Nucleic Acids Research* **41**, Database issue: D590-6.

Rogowski, A., Baslé, A., Farinas, C.S., Solovyova, A., Mortimer, J.C., Dupree, P., Gilbert, H.J. and Bolam, D.N. Evidence That Gh115 A-Glucuronidase Activity, Which Is Required to Degrade Plant Biomass, Is Dependent on Conformational Flexibility. (2014) *The Journal of Biological Chemistry* **289**, 1: 53-64.

Shaufi, M.A.M., Sieo, C.C., Chong, C.W., Gan, H.M. and Ho, Y.W. Deciphering Chicken Gut Microbial Dynamics Based on High-Throughput 16s Rrna Metagenomics Analyses. (2015) *Gut Pathogens* **7**.

Shinkai, T. and Kobayashi, Y. Localization of Ruminal Cellulolytic Bacteria on Plant Fibrous Materials as Determined by Fluorescence in Situ Hybridization and Real-Time Pcr. (2007) *Applied and Environmental Microbiology* **73**, 5: 1646-52.

Simon, M., Grossart, H.P., Schweitzer, B. and Ploug, H. Microbial Ecology of Organic Aggregates in Aquatic Ecosystems. (2002) *Aquatic Microbial Ecology* **28**, 2: 175-211.

Simpson, H.L. and Campbell, B.J. Review Article: Dietary Fibre-Microbiota Interactions. (2015) *Alimentary Pharmacology & Therapeutics* **42**, 2: 158-179.

Stewart, M.L. and Slavin, J.L. Particle Size and Fraction of Wheat Bran Influence Short-Chain Fatty Acid Production *in Vitro*. (2009) *British Journal of Nutrition* **102**, 10: 1404-7.

Torok, V.A., Dyson, C., McKay, A. and Ophel-Keller, K. Quantitative Molecular Assays for Evaluating Changes in Broiler Gut Microbiota Linked with Diet and Performance. (2013) *Animal Production Science* **53**, 12: 1260-1268.

Van Immerseel, F., De Buck, J., Pasmans, F., Velge, P., Bottreau, E., Fievez, V., Haesebrouck, F. and Ducatelle, R. Invasion of *Salmonella* Enteritidis in Avian Intestinal Epithelial Cells in Vitro Is Influenced by Short-Chain Fatty Acids. (2003) *International Journal of Food Microbiology* **85**, 3: 237-48.

Van Immerseel, F., De Zutter, L., Houf, K., Pasmans, F., Haesebrouck, F. and Ducatelle, R. Strategies to Control *Salmonella* in the Broiler Production Chain. (2009) *Worlds Poultry Science Journal* **65**, 3: 367-391.

Van Immerseel, F., Fievez, V., de Buck, J., Pasmans, F., Martel, A., Haesebrouck, F. and Ducatelle, R. Microencapsulated Short-Chain Fatty Acids in Feed Modify Colonization and Invasion Early after Infection with *Salmonella* Enteritidis in Young Chickens. (2004) *Poultry Science* **83**, 1: 69-74.

Van Immerseel, F., Russell, J.B., Flythe, M.D., Gantois, I., Timbermont, L., Pasmans, F., Haesebrouck, F. and Ducatelle, R. The Use of Organic Acids to Combat *Salmonella* in Poultry: A Mechanistic Explanation of the Efficacy. (2006) *Avian Pathology* **35**, 3: 182-8.

Vermeulen, K., Verspreet, J., Courtin, C.M., Haesebrouck, F., Ducatelle, R. and Van Immerseel, F. Reduced Particle Size Wheat Bran Is Butyrogenic and Lowers *Salmonella* Colonization When Added to Poultry Feed. (2017) *Veterinary Microbiology* **198**: 64-71.

Verspreet, J., Damen, B., Broekaert, W.F., Verbeke, K., Delcour, J.A. and Courtin, C.M. A Critical Look at Prebiotics within the Dietary Fiber Concept. (2016) *Annual Review of Food Science and Technology* **7**: 167-90.

Waeonukul, R. *Paenibacillus Curdlanolyticus Strain B-6 Multienzyme Complex: A Novel System for Biomass Utilization*, 2013.

Walker, A.W., Duncan, S.H., Harmsen, H.J., Holtrop, G., Welling, G.W. and Flint, H.J. The Species Composition of the Human Intestinal Microbiota Differs between Particle-Associated and Liquid Phase Communities. (2008) *Environmental Microbiology* **10**, 12: 3275-83.

Wei, S., Morrison, M. and Yu, Z. Bacterial Census of Poultry Intestinal Microbiome. (2013) *Poultry Science* **92**, 3: 671-683.

Wu, Y.B., Ravindran, V., Pierce, J. and Hendriks, W.H. Influence of Three Phytase Preparations in Broiler Diets Based on Wheat or Corn: *In Vitro* Measurements of Nutrient Release. (2004) *International Journal of Poultry Science* **3**, 7: 450-455.

Xu, J., Bjursell, M.K., Himrod, J., Deng, S., Carmichael, L.K., Chiang, H.C., Hooper, L.V. and Gordon, J.I. A Genomic View of the Human-*Bacteroides Thetaiotaomicron* Symbiosis. (2003) *Science* **299**, 5615: 2074-2076.

2

REDUCED PARTICLE SIZE WHEAT BRAN IS BUTYROGENIC AND LOWERS *SALMONELLA* COLONIZATION WHEN ADDED TO POULTRY FEED

Adapted from: Vermeulen K., Verspreet J., Courtin C.M., Haesebrouck F., Baeyen S., Haegeman A., Ducatelle R., Van Immerseel F. (2017). Veterinary Microbiology 198:64-71.

ABSTRACT

Wheat bran is a highly concentrated source of (in)soluble fiber which is partly degraded by the gut microbiota. The aim of the present study was to investigate the potential of wheat bran as such to reduce colonization of the cecum and shedding of *Salmonella* bacteria *in vivo*. Also, the effect of particle size was evaluated. Bran with an average reduced particle size of 280 µm decreased levels of cecal *Salmonella* colonization and shedding shortly after infection when compared to control groups and groups receiving bran with larger particle sizes. *In vitro* fermentation experiments revealed that bran with smaller particle size was fermented more efficiently, with a significantly higher production of butyrate and propionate, compared to the control fermentation and fermentation of the larger fraction. The fermentation products derived from bran with an average particle size of 280 µm downregulated the expression of *hilA*, an important invasion-related gene of *Salmonella*. This downregulation was reflected in an actual lowered invasive potential when *Salmonella* bacteria were pretreated with the fermentation products derived from the smaller bran fraction. These data suggest that wheat bran with reduced particle size can be a suitable feed supplement to combat *Salmonella* infections in broilers. The mechanism of action most probably relies on a more efficient fermentation of this bran fraction and the consequent increased production of SCFA. Among these SCFA, butyrate and propionate are known to reduce the invasion potential of *Salmonella* bacteria.

1. INTRODUCTION

Salmonella enterica is a zoonotic agent of global importance (EFSA 2015). As human salmonellosis is often due to the consumption of contaminated food products of animal origin, control of *Salmonella* in food producing animals is important. Different feed additives have already been investigated to help control *Salmonella* infections, including short- and medium-chain fatty acids such as butyric and caproic acid (Van Immerseel et al. 2004a, Van Immerseel et al. 2006), MOS (Spring et al. 2000, Berge & Wierup 2012), competitive exclusion products (De Cort et al. 2013), and prebiotic DF (Eeckhaut et al. 2008). These DF are carbohydrate polymers that escape digestion in the small intestine and pass into the hindgut where they are (partially) fermented by the microbiota. They can be classified as either being soluble or insoluble (Kumar et al. 2012). Water soluble DF (such as pectins) mostly are fermentable, while insoluble DF (such as cellulose, lignin and hemicellulose) are thought to be less fermentable (Johnson 2001, Stewart & Slavin 2009, Verspreet et al. 2016). The beneficial health effects attributed to the consumption of DF are believed to arise largely from their fermentation by the intestinal microbiota (Nyman et al. 1986). During the fermentation of DF, SCFA are produced of which butyrate, propionate and acetate are the most important ones. The use of DF as feed additive to improve broiler performance has been investigated previously, but the effect of administration of DF on important foodborne pathogens such as *Salmonella* has only been studied to a limited extent. Moreover, the few studies that were conducted only deal with wheat bran-derived purified oligosaccharides. For example, it was shown that the addition of wheat bran-derived AXOS to the feed of broilers can increase the resilience against *Salmonella* (Eeckhaut et al. 2008) and protect against avian coccidiosis (Akhtar et al. 2012).

Wheat bran is an abundantly available by-product from the milling of wheat into flour. It constitutes 14-16% of the wheat kernel and comprises the outer coverings (pericarp), the aleurone layer and remnants of starchy endosperm (Maes & Delcour 2001, Akhtar et al. 2012, Stevenson et al. 2012). Wheat bran is recognized to be a highly concentrated source of insoluble fiber and consists of both fermentable and non-fermentable DF (Leitch et al. 2007). The main DF present are glucuronoarabinoxylans linked to other macromolecules (such as lignin, proteins or both), cellulose, mixed linked (1-3),(1-4)-β-D-glucans, lignin and fructan, constituting respectively 70%, 24%, 1-3%, 3-10% and 3-4% of total wheat bran DF (Maes & Delcour 2002, Hemdane et al. 2016). These AXs are largely water-insoluble (Dervilly et al. 2000). Besides DF, starch

(10-20%), and proteins (15-22%) are the predominant chemical constituents of wheat bran (Maes & Delcour 2001). In humans, the particle size of wheat bran has been shown to influence its physicochemical effects. Indeed, mean digestibility and transit time increases while fecal moisture and stool weight decreases with decreasing particle (Brodribb & Groves 1978, Heller et al. 1980, Jenkins et al. 1999). Up till now, nothing is known about the optimal particle size of DF when administered as feed additive in poultry.

Because wheat bran is a readily available and cheap source of DF and it has been shown that some derivatives of wheat bran can affect *Salmonella* colonization, we evaluated the effect of wheat bran of different particle size as feed additives on colonization and shedding of *Salmonella* in broilers. To our knowledge, this is the first report of the effect of adding wheat bran of different particle size to poultry feed on *Salmonella* colonization.

2. MATERIALS AND METHODS

2.1 BACTERIAL STRAINS

The streptomycin resistant *Salmonella enterica* serotype Enteritidis phage type 4 strain 147 (SE147) was used in both *in vivo* and *in vitro* experiments. This strain was originally isolated from egg white and is known to colonize the chicken gut and internal organs to a high extent (Methner et al. 1995).

2.2 MODIFIED WHEAT BRAN FRACTIONS

The particle size of wheat bran was reduced as described previously (Jacobs et al. 2015). In short, commercial wheat bran (Dossche Mills, Deinze, Belgium) was reduced in particle size with a Cyclotec 1093 Sample mill (FOSS, Höganäs, Sweden). By changing the grinding ring and/or the mesh size of the final sieve of the mill, bran fractions with different particle sizes were obtained. The particle size distribution of the resulting fractions was determined by sieving 20.0 g of bran on a set of sieves with mesh sizes of 4500, 2000, 1000, 710, 500, 400, 250, 200, 160, 125, 112, 90, 50, and 38 µm. The set of sieves was shaken for 30 min at a frequency of 1.5 s^{-1} with a retch Vibratory Sieve Shaker (Aartselaar, Belgium) and each sieve was gently brushed to avoid clogging of the sieve pores. The mass of the bran that remained on each sieve was determined and used to calculate a mass based average particle size:

$$d_{av} = \sum d_i \, . \, m_i$$

with d_i = (upper size limit of bran fraction i + lower limit of bran fraction i)/2 and m_i = mass fraction, i.e. mass on sieve i/sum of masses of all fractions.

Regular wheat bran (1690 µm) and wheat bran with average particle sizes of 1200 µm, 520 µm, 280 µm, 150 µm and 80 µm were used in the experiments described below.

2.3 ANIMALS

One-day-old Ross 308 broilers were obtained from a local hatchery and housed in containers on wood shavings. A commercial standard mash ration (Table 1) and drinking water were provided *ad libitum*. Experiments were performed with the permission of the

Ethical Committee of the Faculty of Veterinary Medicine, Ghent University, Belgium (EC 2014/76 & EC 2014/146).

2.3.1 Experiment 1: The effect of wheat bran with different average particle sizes in a *Salmonella* infection model.

To evaluate the effect of the average particle size of wheat bran on the colonization of *Salmonella* in chickens, 140 one-day-old chicks were divided into 7 groups of 20 animals. The feed of each group was supplemented with 1% of the different wheat bran fractions, i.e. unmodified bran (1690 µm) or bran with an average reduced particle size of 1200 µm, 520 µm, 280 µm, 150 µm or 80 µm. The control group received non-supplemented feed. At the age of 10 days, all chickens were given 10^6 colony forming units (CFU) of the SE147 challenge strain by oral gavage. Shedding of the SE147 challenge strain was monitored by taking cloacal swabs on day 1 post infection (dpi+1) and dpi+3. On dpi+4 the chickens were euthanized and *Salmonella* counts in cecum were determined. Cloacal swabs were also collected on dpi-1 to ensure that all chickens were *Salmonella* negative prior to experimental infection.

Table 1
Nutrient composition of the administered feed.

Nutrient composition	
Crude protein	21.0%
Crude fat	6.0%
Crude ash	5.5%
Crude fibre	3.0%
Lysine	1.17%
Methionine	0.52%
Calcium	0.85%
Phosphorus	0.58%
Sodium	0.15%

2.3.2 Experiment 2: Confirmation of the effects of a wheat bran fraction with an average reduced particle size of 280 µm in a *Salmonella* seeder bird infection model

A seeder bird model was used to evaluate the effect of bran with reduced particle size on *Salmonella* infection in broilers. Seeder bird models allow the infection to spread in a more natural way. One hundred eighty one-day-old chicks were divided into 3 groups of 60 animals. The feed of the animals in the treatment groups was supplemented with 1% unmodified wheat bran (1690 µm) or 1% wheat bran with an average reduced particle size of 280 µm. The control group received non-supplemented feed. On day 10, 12 chickens in each group (one chicken out of five) received 10^8 CFU of the SE147 challenge strain by oral gavage. These seeder birds were marked using leg rings in order to distinguish them from non-seeder birds. Shedding of the SE147 challenge strain was monitored by collecting cloacal

swabs on days dpi+1, dpi+3, dpi+8, dpi+15, dpi+22 and dpi+29. Cloacal swabs were also collected prior to inoculation to verify whether any of the animals was already infected with *Salmonella* prior to onset of the experiment. On day dpi+4, dpi+18 and dpi+32, 20 chickens were euthanized in each group and *Salmonella* counts in cecum were determined.

2.4 BACTERIOLOGICAL ANALYSIS

Cloacal swabs were directly inoculated on Xylose Lysine Deoxycholate agar (XLD, Oxoid, Basingstoke, England) plates supplemented with 100 µg/ml streptomycin (Sigma-Aldrich, St. louis, MO, USA). Negative samples were pre-enriched in buffered-peptone water (BPW, Oxoid, Basingstoke, England) overnight at 37°C. One ml of this suspension was enriched by adding 9 ml of tetrathionate-brilliant green broth (Merck, Darmstadt, Germany). After another overnight incubation at 37°C the suspension was plated onto XLD supplemented with 100 µg/ml streptomycin.

Cecal samples were diluted 10 times and homogenized in BPW, after which 10-fold dilutions were made in phosphate-buffered saline (PBS). For each dilution, 6 droplets were plated onto XLD plates supplemented with 100 µg/ml streptomycin sulfate salt (Sigma-Aldrich). These plates were then incubated overnight at 37°C. The number of CFU/g tissue was determined by counting the number of colonies on the plates. Samples negative after direct plating were enriched as described above. Samples negative after direct plating but positive after enrichment were assumed to contain 1 x 10^1 CFU/g cecum. Samples negative after enrichment were considered to be negative. Bacterial counts were log transformed. Statistical analysis was done with SPSS Statistics 23 (IBM Corp, New York, United States), using a Kruskall-Wallis test, followed by Dunn's post hoc test, to determine statistical differences in the number of *Salmonella* positive cloacal swabs and cecal *Salmonella* counts between groups.

2.5 *IN VITRO* DIGESTION AND FERMENTATION

The different wheat bran fractions were predigested *in vitro* using a protocol previously described by Wu et al. (2004) which was slightly adapted (Wu et al. 2004). First, 1 g substrate was incubated with 1.5 ml 0.03 M HCl (40°C for 30 min) to mimic the initial stages of digestion in the crop. Secondly, the digestion in the proventriculus and gizzard was

simulated by incubating the substrate with 3000 U of pepsin from porcine gastric mucosa (Sigma-Aldrich, St. Louis, United States) in 1 ml 1.5 M HCl (40°C for 45 min). To simulate digestion in the duodenum, 1 ml 1 M $NaHCO_3$ and 3.7 mg pancreatin from porcine pancreas (Sigma-Aldrich) were added (2 h at 40°C). Subsequently the predigested bran fractions were centrifuged (5 min, 5000xg) and washed twice with 20 ml of aqua dest. The resulting pellet was retained and lyophilized. The *in vitro* fermentations were performed using a nutrient-poor medium described by Moura et al. (2007) with minor modifications, previously described by De Maesschalck et al. (2015) (Moura et al. 2007, De Maesschalck et al. 2015). The pH of the medium was adjusted to 6.5. The ceca of 4-week old Ross 308 broilers were isolated and brought immediately into an anaerobic cabinet (Ruskinn technology, Bridgend, United Kingdom) with 84% N_2, 8% H_2 and 8% CO_2 at 37°C. The lyophilized predigested fractions were supplemented to the nutrient-poor medium to a concentration of 1% (w/v). Non-supplemented medium was used as a control. After 24 h anaerobic incubation at 37°C and centrifugation, the supernatant was collected (2500xg, 10 min). The experiment was repeated three times using cecal contents obtained from three different chickens.

2.6 SCFA QUANTIFICATION

The method previously described by De Weirdt et al. (2010) was used to quantify the amounts of butyrate, propionate and acetate (De Weirdt et al. 2010). In short, SCFA were extracted from the samples using diethyl ether. Methyl hexanoic acid 99% (Sigma-Aldrich) was added as internal standard. The extracts were analyzed using a GC-2014 gas chromatograph (Shimadzu,'s-Hertogenbosch, the Netherlands), equipped with a capillary fatty acid-free EC-1000 Econo-Cap column (dimensions: 25mm x 0.53 mm, film thickness 1.2 mM; Alltech, Laarne, Belgium), a flame ionization detector and a split injector. The injection volume was 1 µL and the temperature profile was set from 110 to 160°C, with a temperature increase of 6 °C/min The carrier gas was nitrogen and the temperature of the injector and detector were 100 and 220 °C, respectively. Statistical analysis was carried out with SPSS Statistics 23, using a one factor ANOVA with Tukey post-hoc test.

2.7 MEASUREMENT OF *HILA* EXPRESSION

To determine the expression of the *hilA* gene, the promotor of *hilA* was cloned in front of *luxCDABE* genes. The production of light is proportional to the expression level of the gene,

which was determined relatively to the expression of the housekeeping gene *rpsM*. The construction of a SE147 strain carrying a *hilA*-lux promoter fusion was performed as described previously (Van Immerseel et al. 2004a). A *rpsM* promoter fusion was constructed similarly by using *rpsM* specific primers NNNNCTCGAGATCGTTAAGCGTGATGG/ NNNNGGATCCCGACACCGTAGATCGAAG for the initial amplification of the *rpsM* promoter sequence. This resulted in cloning a sequence 214 bp upstream from the *rpsM* start codon containing *rpsM* promoter into pCS26, upstream from the lux reporter genes.

To quantify light production by a SE147 strain carrying plasmids either containing *hilA-luxCDABE* or *rpsM-luxCDABE* transcriptional fusions, a Fluoroscan Ascent fluorometer (Lab-Systems, Helsinki, Finland) was used. Overnight cultures of SE147 carrying either of the reporter fusion constructs were diluted 1:100 in Luria Bertani broth (LB, Sigma-Aldrich), supplemented with supernatant (1:1) derived from the different *in vitro* fermentation reactions. As a control, LB (Sigma-Aldrich) was supplemented with supernatant (1:1) derived from the control fermentation. Subsequently the diluted bacterial cultures were grown in microplates at 37°C. Light production was measured automatically every 15 min during 15 hours. Statistical analysis was carried out with SPSS Statistics 23, using a two factor ANOVA with chicken as random factor, followed by a Tukey post hoc-test.

2.8 INVASION ASSAY

Cells of the human colon carcinoma cell line T84 were seeded in 96-well cell culture plates at a density of 5 x 10^5 cells/ml cell culture medium (Dulbecco's modified Eagle's medium (DMEM), 10% fetal calf serum (FCS), and 2% L-glutamine) and grown for 24 hours at 37°C and 5% CO_2. SE 147 was grown overnight at 37°C in LB medium (Sigma-Aldrich), after which the medium was diluted 1:100 in LB medium (Sigma-Aldrich) supplemented with supernatant (1:1) obtained from the different fermentation reactions. After 16 hours the bacterial cultures were centrifuged (10 min, 5200xg) and the LB medium (Sigma-Aldrich) was replaced by cell culture medium. The suspensions were diluted to a density of 10^6 CFU/ml. Two hundred µl of this dilution was transferred onto the T84 cells. Contact between bacterial cells and human cells was achieved by centrifugation (10 min, 525xg). Thereafter, inoculated cell cultures were incubated at 37°C and 5% CO_2. After 1h the T84 cells were rinsed 3 times with Hanks' Balanced Salt Solution (HBSS, Life Technologies, Paisley, Scotland). Cell medium

with 100 µg/ml gentamicin was added and the plates were incubated again for 1 hour at 37°C and 5% CO_2. Next, the cells were rinsed 5 times with HBSS (Life Technologies, Paisley, Scotland) and lysed with 0.2% sodium deoxycholate (Sigma) in distilled water. The number of CFU/ml was determined by plating 6 x 20 µl of a 10-fold dilution series of the suspensions onto XLD agar (Oxoid) plates. Invasion was calculated as the percent invaded bacteria relative to the initial number of inoculated bacteria. Statistical analysis was carried out with SPSS Statistics 23, using a two factor ANOVA with chicken as random factor, followed by a Tukey post hoc-test.

3. RESULTS

3.1 WHEAT BRAN WITH AVERAGE REDUCED PARTICLE SIZE OF 280 µM REDUCES CECAL COLONIZATION BY *SALMONELLA* ENTERITIDIS SE147 SHORTLY AFTER INFECTION

During the first *in vivo* experiment, broiler chickens were fed diets supplemented with 1% of different wheat bran fractions and were experimentally infected with SE147. Before infection all chickens tested negative for *Salmonella*. On dpi+1 and dpi+3 cloacal swabs were collected to monitor shedding. All groups shed SE147 to similar levels during the experiment, as no statistical significant differences were observed between the different groups (Table 2). On dpi+4, all animals were euthanized and bacterial counts in the cecum were performed. Animals fed wheat bran with reduced average particle size of 280 µm, displayed a significantly lower cecal colonization compared to the control group (p=0.036) and the group receiving bran with reduced particle size of 520 µm (p=0.003) (Table 3). In general, a higher number of *Salmonella* negative ceca were found in groups fed bran with smaller particle sizes (280 µm, 150 µm and 80 µm). Moreover, the number of *Salmonella* negative ceca was significantly higher for the group receiving bran with reduced particle size of 280 µm compared to the control group (p=0.006) (Figure 1).

Table 2
Fecal shedding of *Salmonella* Enteritidis strain SE147 after experimental infection of broilers fed a diet containing different wheat bran fractions.

	dpi-1	dpi+1	dpi+3
Control	0/20	5/20	8/20
1690 µm	0/20	7/20	11/18
1200 µm	0/20	7/20	6/19
520 µm	0/20	2/20	4/19
280 µm	0/20	4/20	6/19
150 µm	0/20	8/20	6/20
80 µm	0/20	6/20	6/19

Chickens were orally inoculated with 10^6 CFU of *Salmonella* Entertitidis strain SE147 when they were 10 days old. Cloacal swabs were collected at dpi-1, dpi+1 and dpi+3. Table shows the number of *Salmonella* shedding animals per group.

Table 3
Cecal colonization by *Salmonella* Enteritidis (SE147) on dpi+4.

	Mean ± SD	p-value
Control	3.6±1.5[a]	p<0.05
1690 µm	3.1±1.8[ab]	
1200 µm	3.4±2.2[ab]	
520 µm	4.1±1.8[a]	p<0.01
280 µm	1.6±1.9[b]	
150 µm	2.9±2.5[ab]	
80 µm	2.2±2.1[ab]	

Mean log CFU/ g cecum values and standard deviations are shown. Significant differences among groups are indicated with different letters ([a], [b]).

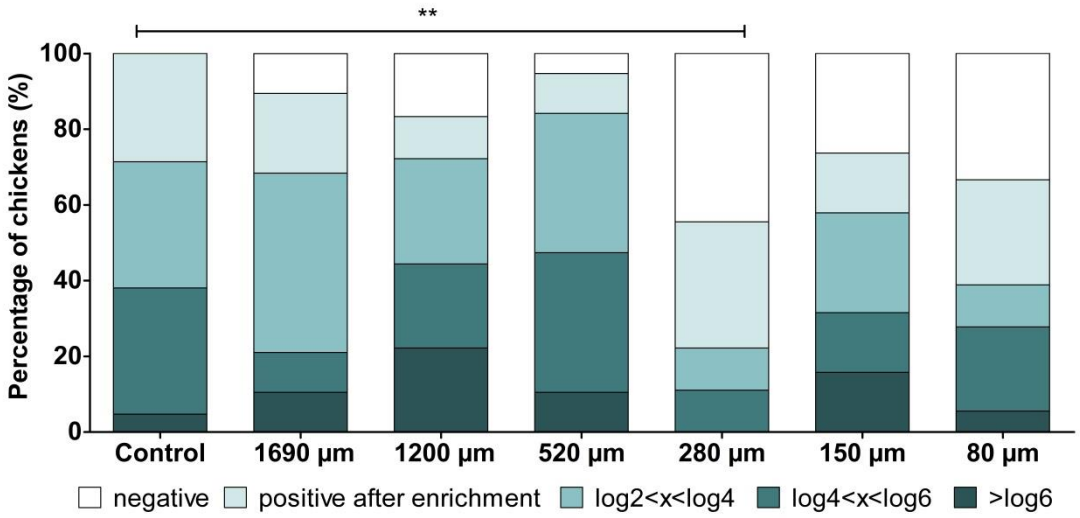


Figure 1 • Cecal colonization by *Salmonella* Enteritidis (SE147). All chickens were inoculated with 10^6 CFU *Salmonella* Enteritidis (SE147) on day 10 of the experiment. On dpi+4, the chickens were euthanized and bacterial counts in cecum were determined. The different bran fractions are depicted on the x-axis while the y-axis shows the percentage of chickens containing a specified log number of CFU per gram cecum. Significant differences in numbers of *Salmonella* negative chickens are indicated with **0.01≤p≤0.001.

3.2 EXPERIMENT 2: CONFIRMATION OF THE EFFECTS OF WHEAT BRAN WITH AN AVERAGE REDUCED PARTICLE SIZE OF 280 µM IN A *SALMONELLA* SEEDER BIRD INFECTION MODEL

Shedding of *Salmonella* was monitored by collecting cloacal swabs on dpi-1, dpi+1, dpi+3, dpi+8, dpi+15, dpi+22 and dpi+29. On dpi+3 and dpi+8, animals fed bran with reduced particle size (280 µm), shed significantly less of the SE147 than the group receiving unmodified bran (1690 µm) (p<0.001 and p=0.003, respectively). In addition, on dpi+8, animals receiving 280 µm bran shed significantly less compared to the control group (p<0.001) (Table 4). On dpi+4, dpi+18 and dpi+32, 20 chickens were euthanized per group

and *Salmonella* counts in cecum were performed by bacteriological analysis. On dpi+4, the mean number of *Salmonella* bacteria present in the cecal contents was significantly lower in the group of animals receiving bran with reduced particle size (280 µm) when compared to the control group (p=0.028) and the group receiving unmodified bran (1690 µm) (p=0.015) (Table 5). On dpi+4, the number of *Salmonella* negative chickens was significantly lower for the group receiving bran with reduced particle size of 280 µm compared to the control group (p=0.013) and the group receiving unmodified bran (1690 µm) (p<0.001). On dpi+22, significantly more negative samples were found in the control group when compared to the group receiving unmodified bran (1690 µm) (p<0.001) (Figure 2).

Table 4

Fecal shedding of *Salmonella* Enteritidis strain SE147 after experimental infection of broilers fed with different wheat bran fractions.

	dpi-1	dpi+1	dpi+3	dpi+8	dpi+15	dpi+22	dpi+29
Control	0/60	15/60	16/59	23/40	20/40	7/19	6/19
1690 µm	0/60	19/60	31/60	21/40	27/39	8/20	3/19
280 µm	0/60	8/60	11/60	8/40	25/40	10/20	6/20
p-value			p<0.001	p<0.01			

One chicken out of five was inoculated with 10^8 CFU of Salmonella Entertitidis SE147 when they were 10 days old. Cloacal swabs were collected on days post infection (dpi) dpi-1, dpi+1, dpi+3, dpi+8, dpi+15, dpi+22 and dpi+29. Table shows the number of *Salmonella* shedding animals per group. Significant differences among groups within one sampling day are indicated with different letters (a, b).

Table 5

Cecal colonization by *Salmonella* Enteritidis (SE147) on dpi+4, dpi+18 and dpi+32.

dpi+4	Mean ± SD	p-value
Control	3.6 ± 2.4^b	p<0.05
1690 µm	3.7 ± 1.9^b	p<0.05
280 µm	1.3 ± 2.2^a	
dpi+18	Mean ± SD	
Control	2.2±1.0	
1690 µm	2.9±1.7	
280 µm	2.5±0.6	
dpi+32	Mean ± SD	
Control	0.1±0.6	
1690 µm	1.3±1.0	
280 µm	1.2±1.4	

Table shows mean log CFU/ g cecum values ± standard deviations.
Significant differences among groups are indicated with different letters (^{a}, ^{b}).

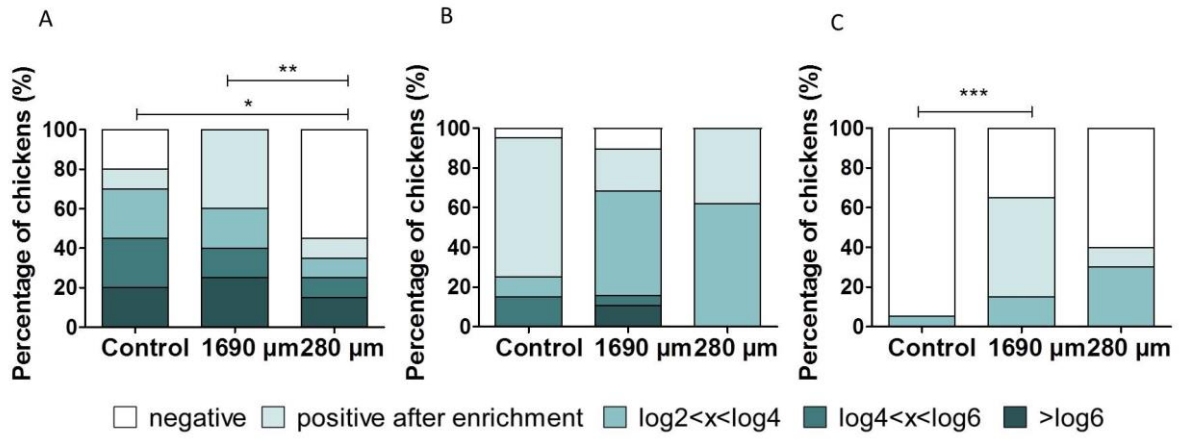


Figure 2 • Cecal colonization by *Salmonella* Enteritidis (SE147). In every group, one chicken in five (n=12) was inoculated with 10^8 CFU of SE147 when they were 10 days old. On dpi+4 (A), dpi+18 (B) and dpi+32 (C), 20 chickens per group were euthanized and bacterial counts in the ceca were determined. The different bran fractions are depicted on the x-axis while the y-axis shows the percentage of chickens containing a specified log number of CFU per gram cecum. Significant differences within sampling days in numbers of *Salmonella* negative chickens are indicated with *0.01<p<0.05; **0.001≤p≤0.01; ***p<0.001

3.3 *IN VITRO* FERMENTATION OF WHEAT BRAN WITH REDUCED PARTICLE SIZE LEADS TO AN INCREASED SCFA PRODUCTION

Wheat bran fractions with an average particle size of 280 and 1690 µm were fermented in a basal medium for 24h using chicken cecal contents. Afterwards the supernatant was collected in order to quantify the amounts of acetate, propionate and butyrate produced. The fermentation of unmodified bran (1690 µm) and bran with reduced particle size (280 µm) resulted in higher amounts of total SCFA when compared to the control fermentation (p=0.008, p=0.001 respectively). In addition, fermentation of bran with reduced particle size (280 µm) resulted in significantly higher levels of total SCFA compared to the unmodified fraction (1690 µm) (p=0.042) (Figure 3 A). Both the fermentation of unmodified bran (1690 µm) and the particle size reduced fraction (280 µm) resulted in the production of significantly more butyrate (p=0.004, p=0.001 respectively) compared to the control fermentation (Figure 3 D). The fermentation of bran with average particle size of 280 µm resulted in a significantly higher concentration of acetate compared to the control fermentation (p=0.04). Propionate was produced in significantly higher amounts during the fermentation of bran with reduced particle size (280 µm) compared to the control fermentation (p=0.042).

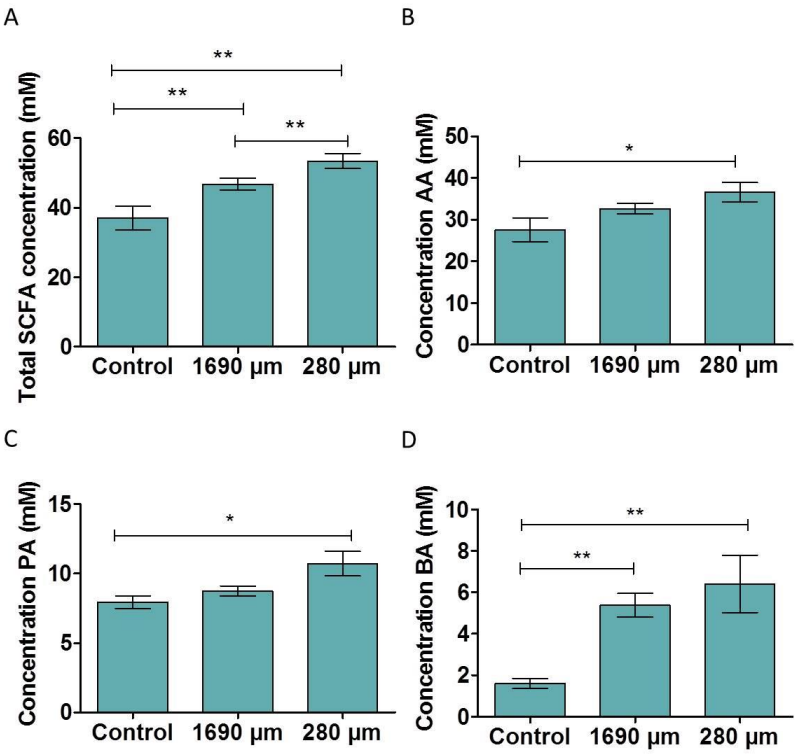


Figure 3 • Amounts of total SCFA (A), acetate (B), propionate (C) and butyrate (D) produced during *in vitro* fermentations of wheat bran with different particle sizes. Supernatant of the *in vitro* fermentations was collected after 24h. Total amount of SCFA was calculated as the sum of acetate, propionate and butyrate. Bars represent the mean, error bars represent the standard deviation. AA: acetate, PA: propionate, BA: butyrate. *0.01<p<0.05; **0.001≤p≤0.01

3.4 FERMENTATION PRODUCTS OF WHEAT BRAN WITH REDUCED PARTICLE SIZE OF 280 µM DOWNREGULATE *HILA* EXPRESSION

Stationary SE147 cultures carrying a plasmid containing either *hilA-luxCDABE* or *rpsM-luxCDABE* transcriptional fusions were diluted in LB medium supplemented with supernatant (1:1) derived from the fermentation in which respectively, no bran (control), unmodified bran (1690 µm) or bran with reduced particle size (280 µm) was added. Two effects were observed. First, bacteria grown in the presence of fermentation products derived from unmodified bran (1690 µm) or particle size reduced bran (280 µm) showed a reduced *hilA* expression (p=0.021 and p<0.001 respectively) compared to the control. In addition, bacteria grown in the presence of fermentation products derived from bran with reduced particle size (280 µm) showed a significant decrease in *hilA* expression when compared to the SE147 strain grown in the presence of fermentation products derived from unmodified bran (1690 µm) (p=0.002) (Figure 4).

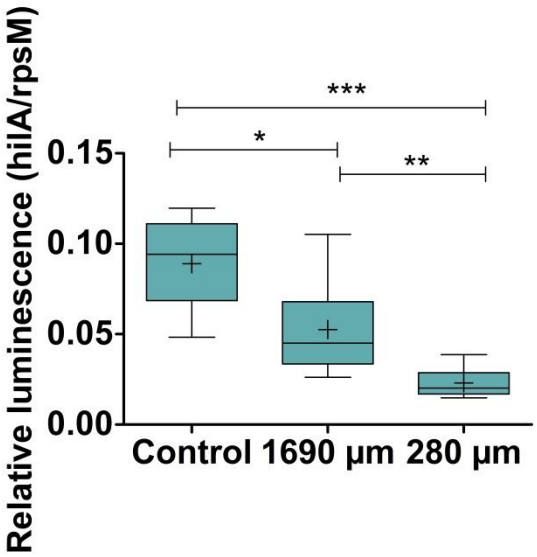


Figure 4 • Relative expression of the *hilA* gene after exposure of *Salmonella* Enteritidis *hilA*-lux or *rpsM*-lux fusion construct strains to *in vitro* fermented wheat bran fractions (y-axis). Overnight cultures *of Salmonella* Enteritidis strain SE147 carrying a plasmid containing either *hilA*-luxCDABE or *rpsM*-luxCDABE transcriptional fusions were diluted in LB medium supplemented with supernatant derived from respectively the fermentation without bran (control), with unmodified bran (1690 µm) and bran with reduced particle size (280 µm) (x-axis). The diluted cultures were grown in microplates at 37°C for 15 hours. The plus represents the mean value, whiskers are the median, the min/max value and $1^{ste}/3^{rd}$ quartiles. Data represents the measurement after 15 hours of incubation. *0.01<p<0.05; **0.001≤p≤0.01; *** p<0.001

3.5 INVASION IN EPITHELIAL CELLS IS REDUCED *IN VITRO* WHEN *SALMONELLA* IS PRETREATED WITH FERMENTATION PRODUCTS DERIVED FROM WHEAT BRAN WITH REDUCED PARTICLE SIZE

In order to investigate the effect of a diet supplemented with different wheat bran fractions on the ability of *Salmonella* Enteritidis to invade host cells, an invasion assay was performed using *Salmonella* Enteritidis cultures exposed to fermentation products derived from unmodified bran (1690 µm), bran with an average reduced particle size of 280 µm, or the control fermentation. Bran size had a significant effect on the percentage of invading bacteria as pretreatment with fermentation products derived from bran with reduced particle size (280 µm) resulted in a relative reduction in percentage invasion of 63% (p=0.019) when compared to the invasion of *Salmonella* pretreated with products obtained from the control fermentations. Pretreatment with fermentation products derived from

bran with average reduced particle size of 280 µm resulted in a relative reduction in percentage invasion of 36% (p=0.002), compared to *Salmonella* pretreated with products obtained from the fermentation of unmodified bran (1690 µm) (Figure 5).

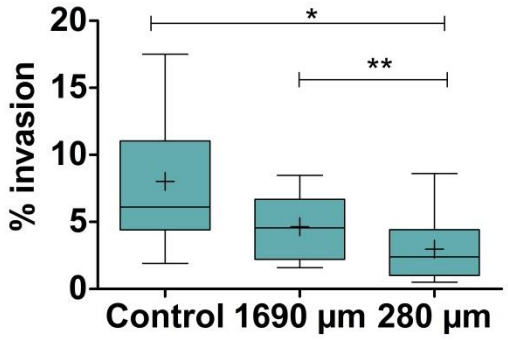


Figure 5 • Effect of particle size on the ability of *Salmonella* to invade host cells. *Salmonella* Enteritidis bacteria were pre-incubated in LB medium supplemented with supernatant derived from one of three fermentation reactions in which no bran (control), unmodified bran (1690 µm) and bran with reduced particle size (280 µm) were added. Hereafter an invasion assay was performed. Invasion was calculated as the percent invading bacteria relative to the initial number of inoculated bacteria (y-axis). The different bran fractions are depicted on the x-axis. The plus represents the mean value, whiskers are the median, the min/max value and $1^{ste}/3^{rd}$ quartiles. *0.01<p<0.05; **0.001≤p≤0.01

4. DISCUSSION

In 2014 in the E.U., the prevalence of broiler flocks positive for *Salmonella* was 3.4%. The most common serovar was *Salmonella* Enteritidis (Authority et al. 2015). Since the EU *Salmonella* control target is defined as a maximum percentage of broiler flocks remaining positive of 1% or less, there is still work to be done. Several approaches using feed additives have already been applied to control *Salmonella* in broilers. For example, we previously showed that wheat bran derived AXOS decrease colonization and shedding of *Salmonella* Enteritidis in broilers (Eeckhaut et al. 2008). Since AXs are the main DFs present in wheat bran, we investigated the potential of wheat bran as such to reduce colonization of the cecum and shedding of *Salmonella* bacteria in different infection models. Also, the effect of particle size of the bran was evaluated.

Wheat bran with reduced particle size has potential as a feed additive in broilers because of the inhibitory effects on *Salmonella*. Indeed, in our study, in two distinct infection models, bran with reduced particle size (280 µm) decreased levels of cecal *Salmonella* colonization and shedding of bacteria shortly after infection compared to control groups and groups receiving bran with larger particle sizes. This effect seemed to fade in time. It is possible that initial colonization is impaired and infection is delayed but once colonization is established, infection can spread unequivocally. However, infection levels at the end of the trial may have been too low to draw a proper conclusion on this aspect.

Although *Salmonella* is an important pathogen in the broiler industry, feed additives are generally more frequently used when they also have benefits on broiler performance. It has been shown that particle size of DF sources plays a pivotal role in broiler performance. Indeed, Rezaei et al. (2011) showed that Vitacel, a micronized commercial by-product of wheat, is able to increase daily body weight gain and improve feed conversion in broilers (Rezaei et al. 2011). Jimenez-Moreno et al. (2010) reported similar effects using oat hulls with reduced particle size (Jimenez-Moreno et al. 2010).

The more efficient fermentation of smaller bran fractions is likely to be caused by increased accessibility for bacterial enzymes. When fermenting the different bran fractions *in vitro*, the fraction with reduced particle size (280 µm) produced the highest levels of total SCFA. This is in accordance with the results obtained by Stewart & Slavin (2009), who fermented coarse

whole-wheat bran (1239 µm) and fine whole-wheat bran (551 µm), using human fecal matter (Stewart & Slavin 2009). The enhanced fermentation of bran with smaller particle size is most likely due to the increased accessible surface area as particle size decreases (Jacobs et al. 2015). This way, bacterial enzymes have a larger contact area to access fermentable carbohydrates (Stewart & Slavin 2009). Thomas et al. (2013) confirmed this hypothesis during solid state fermentation experiments. They state that there is a certain optimum concerning particle size: larger particles are less easily accessible and thus less easily broken down by the bacterial community. On the other hand, particles that are too small, hamper fermentation because of agglomeration and consequently lowered microbial respiration. In the present study, there are indications suggesting a similar optimum particle size as further reduction in particle size, below 280 µm, tended to result in higher *Salmonella* counts. Moisture content is another crucial factor during fermentation (Thomas et al. 2013). Jacobs et al. (2015) showed that particle size can influence the hydration properties of wheat bran. They demonstrated that the water absorption capacity decreases with particle size (Jacobs et al. 2015). So it can be expected that there is some kind of optimal particle size that positively influences fermentation.

It is hypothesized that the decrease in colonization by *Salmonella* when animals were fed wheat bran with reduced particle size is caused by effects of fermentation products on invasion of *Salmonella*. Invasion of *Salmonella* into intestinal epithelial cells is regulated by genes of the *Salmonella* pathogenicity island I (SPI-1) (Galan & Curtiss 1990). *HilA* is an important transcriptional activator of the SPI1 cluster of invasion genes in *Salmonella*. Its importance for entry in epithelial cells has been demonstrated by creating a null mutation in the *hilA* gene, causing a dramatic attenuation of invasion and consequently gut colonization (Fahlen et al. 2000). The expression of *hilA* is tightly regulated by both regulatory and environmental factors, the latter including osmolarity, oxygen, and pH (Bajaj et al. 1996). Previous studies showed that butyrate, already at low concentrations, can decrease the expression of *hilA* (Van Immerseel et al. 2003, Van Immerseel et al. 2004b, Gantois et al. 2006, Boyen et al. 2008). Furthermore Durant et al. (1999, 2000) showed that propionate could modulate the expression of *hilA* as well, and consequently lower the invasion potential of *Salmonella* Typhimurium *in vitro* (Durant et al. 1999, Durant et al. 2000). In our study we showed that fermentation products derived from the different bran fractions could induce a downregulation of the *hilA* gene. This downregulation

resulted in a lowered invasion of *Salmonella* bacteria that were pretreated with fermentation products of bran with reduced particle size. Both the fermentation of unmodified bran and bran with reduced particle size (280 µm) produced higher levels of butyrate compared to the control fermentation. The fermentation of bran with an average particle size of 280 µm resulted in a significantly higher concentration of propionate. Most probably, the (combined) increased production of butyrate and propionate during fermentations, is involved in reduced *hilA* expression, reduced invasive potential and consequently lower cecal colonization.

In conclusion, wheat bran with reduced particle size may be a suitable feed supplement to help control *Salmonella* infections in broilers since the addition of concentrations as low as 1% already significantly reduced cecal colonization and fecal shedding shortly after infection. The mechanism of action most probably relies on a more efficient fermentation of bran with reduced particle size in the cecum and the consequent increased production of SCFA. Butyrate and propionate are able to downregulate important invasion genes of *Salmonella* bacteria which eventually may lead to a decreased invasion of epithelial cells and an increased resilience of chickens against *Salmonella* infections. Further investigation is needed to verify whether, besides butyrate and propionate, other unidentified compounds are involved.

CONFLICT OF INTEREST STATEMENT

K. Vermeulen, J. Verspreet, C.M. Courtin, F. Van Immerseel and R. Ducatelle are listed as coinventors on a patent application for a wheat bran fraction for use to control *Salmonella* infection (International Application Number PCT/EP2017/067028).

ACKNOWLEDGMENTS

The authors would like to thank Dr. Ivan Rychlik (Veterinary Research Institute, Brno, Czech Republic) for providing the *hilA*- and *rpsM*-containing plasmids. We would also like to thank Petra Van Damme and Isabel Lemahieu for their kind assistance during osmolarity adjustments and Herman De Rycke for lyofilisation. We thank the “Vlaams Agentschap Innoveren en Ondernemen” (Vlaio, former IWT or “Agentschap voor Innovatie door Wetenschap en Technologie ”) for the financial support of the SBO BRANDING project (grant no. 130028). The authors acknowledge the PhD students from the department of Pathology, Bacteriology and Avian Diseases who assisted in the sampling of the in *vivo* trial.

REFERENCES

Akhtar, M., Tariq, A.F., Awais, M.M., Iqbal, Z., Muhammad, F., Shahid, M. and Hiszczynska-Sawicka, E. Studies on Wheat Bran Arabinoxylan for Its Immunostimulatory and Protective Effects against Avian Coccidiosis. (2012) *Carbohydrate Polymers* **90**, 1: 333-9.

Authority, E.F.S., Prevention, E.C.f.D. and Control. The European Union Summary Report on Trends and Sources of Zoonoses, Zoonotic Agents and Food-Borne Outbreaks in 2014. (2015) *EFSA Journal* **13**, 12.

Bajaj, V., Lucas, R.L., Hwang, C. and Lee, C.A. Co-Ordinate Regulation of *Salmonella* Typhimurium Invasion Genes by Environmental and Regulatory Factors Is Mediated by Control of *Hila* Expression. (1996) *Molecular Microbiology* **22**, 4: 703-14.

Berge, A.C. and Wierup, M. Nutritional Strategies to Combat *Salmonella* in Mono-Gastric Food Animal Production. (2012) *Animal* **6**, 4: 557-64.

Boyen, F., Haesebrouck, F., Vanparys, A., Volf, J., Mahu, M., Van Immerseel, F., Rychlik, I., Dewulf, J., Ducatelle, R. and Pasmans, F. Coated Fatty Acids Alter Virulence Properties of *Salmonella* Typhimurium and Decrease Intestinal Colonization of Pigs. (2008) *Veterinary Microbiology* **132**, 3-4: 319-27.

Brodribb, A.J. and Groves, C. Effect of Bran Particle Size on Stool Weight. (1978) *Gut* **19**, 1: 60-3.

De Cort, W., Geeraerts, S., Balan, V., Elroy, M., Haesebrouck, F., Ducatelle, R. and Van Immerseel, F. A *Salmonella* Enteritidis *Hilassraflig* Deletion Mutant Is a Safe Live Vaccine Strain That Confers Protection against Colonization by *Salmonella* Enteritidis in Broilers. (2013) *Vaccine* **31**, 44: 5104-10.

De Maesschalck, C., Eeckhaut, V., Maertens, L., De Lange, L., Marchal, L., Nezer, C., De Baere, S., Croubels, S., Daube, G., Dewulf, J., Haesebrouck, F., Ducatelle, R., Taminau, B. and Van Immerseel, F. Effects of Xylo-Oligosaccharides on Broiler Chicken Performance and Microbiota. (2015) *Applied Environmental Microbiology* **81**, 17: 5880-5888.

De Weirdt, R., Possemiers, S., Vermeulen, G., Moerdijk-Poortvliet, T.C.W., Boschker, H.T.S., Verstraete, W. and Van de Wiele, T. Human Faecal Microbiota Display Variable Patterns of Glycerol Metabolism. (2010) *FEMS Microbiology Ecology* **74**, 3: 601-611.

Dervilly, G., Saulnier, L., Roger, P. and Thibault, J. Isolation of Homogeneous Fractions from Wheat Water-Soluble Arabinoxylans. Influence of the Structure on Their Macromolecular Characteristics. (2000) *Journal of Agricultural and Food Chemistry* **48**, 2: 270-8.

Durant, J.A., Corrier, D.E. and Ricke, S.C. Short-Chain Volatile Fatty Acids Modulate the Expression of the *Hila* and *Invf* Genes of *Salmonella* Typhimurium. (2000) *Journal of Food Protection* **63**, 5: 573-8.

Durant, J.A., Lowry, V.K., Nisbet, D.J., Stanker, L.H., Corrier, D.E. and Ricke, S.C. Short-Chain Fatty Acids Affect Cell-Association and Invasion of Hep-2 Cells by *Salmonella* Typhimurium. (1999) *Journal of Environmental Science and Health* **34**, 6: 1083-99.

Eeckhaut, V., Van Immerseel, F., Dewulf, J., Pasmans, F., Haesebrouck, F., Ducatelle, R., Courtin, C.M., Delcour, J.A. and Broekaert, W.F. Arabinoxylooligosaccharides from Wheat Bran Inhibit *Salmonella* Colonization in Broiler Chickens. (2008) *Poultry Science* **87**, 11: 2329-34.

EFSA. The 2013 Joint Ecdc/Efsa Report on Trends and Sources of Zoonoses, Zoonotic Agents and Food-Borne Outbreaks Published. (2015) *EuroSurveillance* **20**, 4.

Fahlen, T.F., Mathur, N. and Jones, B.D. Identification and Characterization of Mutants with Increased Expression of *Hila*, the Invasion Gene Transcriptional Activator of *Salmonella* Typhimurium. (2000) *FEMS Immunology & Medical Microbiology* **28**, 1: 25-35.

Galan, J.E. and Curtiss, R., 3rd. Expression of *Salmonella* Typhimurium Genes Required for Invasion Is Regulated by Changes in DNA Supercoiling. (1990) *Infection and Immunity* **58**, 6: 1879-85.

Gantois, I., Ducatelle, R., Pasmans, F., Haesebrouck, F., Hautefort, I., Thompson, A., Hinton, J.C. and Van Immerseel, F. Butyrate Specifically Down-Regulates *Salmonella* Pathogenicity Island 1 Gene Expression. (2006) *Applied and Environmental Microbiology* **72**, 1: 946-9.

Heller, S.N., Hackler, L.R., Rivers, J.M., Van Soest, P.J., Roe, D.A., Lewis, B.A. and Robertson, J. Dietary Fiber: The Effect of Particle Size of Wheat Bran on Colonic Function in Young Adult Men. (1980) *American Journal of Clinical Nutrition* **33**, 8: 1734-44.

Hemdane, S., Jacobs, P.J., Dornez, E., Verspreet, J., Delcour, J.A. and Courtin, C.M. Wheat (Triticum Aestivum L.) Bran in Bread Making: A Critical Review. (2016) *Comprehensive Reviews in Food Science and Food Safety* **15**, 1: 28-42.

Jacobs, P.J., Hemdane, S., Dornez, E., Delcour, J.A. and Courtin, C.M. Study of Hydration Properties of Wheat Bran as a Function of Particle Size. (2015) *Food Chemistry* **179**: 296-304.

Jenkins, D.J., Kendall, C.W., Vuksan, V., Augustin, L.S., Li, Y.M., Lee, B., Mehling, C.C., Parker, T., Faulkner, D., Seyler, H., Vidgen, E. and Fulgoni, V. The Effect of Wheat Bran Particle Size on Laxation and Colonic Fermentation. (1999) *Journal of the American College of Nutrition* **18**, 4: 339-45.

Jimenez-Moreno, E., Gonzalez-Alvarado, J.M., Gonzalez-Sanchez, D., Lazaro, R. and Mateos, G.G. Effects of Type and Particle Size of Dietary Fiber on Growth Performance and Digestive Traits of Broilers from 1 to 21 Days of Age. (2010) *Poultry Science* **89**, 10: 2197-212.

Johnson, I.T. New Food Components and Gastrointestinal Health. (2001) *Proceedings of the Nutrition Society* **60**, 4: 481-8.

Kumar, V., Sinha, A.K., Makkar, H.P., de Boeck, G. and Becker, K. Dietary Roles of Non-Starch Polysaccharides in Human Nutrition: A Review. (2012) *Critical Reviews in Food Science and Nutrition* **52**, 10: 899-935.

Leitch, E.C.M., Walker, A.W., Duncan, S.H., Holtrop, G. and Flint, H.J. Selective Colonization of Insoluble Substrates by Human Faecal Bacteria. (2007) *Environmental Microbiology* **9**, 3: 667-679.

Maes, C. and Delcour, J.A. Alkaline Hydrogen Peroxide Extraction of Wheat Bran Non-Starch Polysaccharides. (2001) *Journal of Cereal Science* **34**, 1: 29-35.

Maes, C. and Delcour, J.A. Structural Characterisation of Water-Extractable and Water-Unextractable Arabinoxylans in Wheat Bran. (2002) *Journal of Cereal Science* **35**, 3: 315-326.

Methner, U., Alshabibi, S. and Meyer, H. Experimental Oral Infection of Specific Pathogen-Free Laying Hens and Cocks with *Salmonella* Enteritidis Strains. (1995) *Journal of veterinary medicine. B, Infectious diseases and veterinary public health* **42**, 8: 459-469.

Moura, P., Barata, R., Carvalheiro, F., Girio, F., Loureiro-Dias, M.C. and Esteves, M.P. *In Vitro* Fermentation of Xylo-Oligosaccharides from Corn Cobs Autohydrolysis by *Bifidobacterium* and *Lactobacillus* Strains. (2007) *Food Science and Technology* **40**, 6: 963-972.

Nyman, M., Asp, N.G., Cummings, J. and Wiggins, H. Fermentation of Dietary Fibre in the Intestinal Tract: Comparison between Man and Rat. (1986) *British Journal of Nutrition* **55**, 3: 487-96.

Rezaei, M., Karimi Torshizi, M.A. and Rouzbehan, Y. The Influence of Different Levels of Micronized Insoluble Fiber on Broiler Performance and Litter Moisture. (2011) *Poultry Science* **90**, 9: 2008-12.

Spring, P., Wenk, C., Dawson, K.A. and Newman, K.E. The Effects of Dietary Mannaoligosaccharides on Cecal Parameters and the Concentrations of Enteric Bacteria in the Ceca of Salmonella-Challenged Broiler Chicks. (2000) *Poultry Science* **79**, 2: 205-11.

Stevenson, L., Phillips, F., O'Sullivan, K. and Walton, J. Wheat Bran: Its Composition and Benefits to Health, a European Perspective. (2012) *International Journal of Food Sciences and Nutrition* **63**, 8: 1001-1013.

Stewart, M.L. and Slavin, J.L. Particle Size and Fraction of Wheat Bran Influence Short-Chain Fatty Acid Production *in Vitro*. (2009) *British Journal of Nutrition* **102**, 10: 1404-7.

Thomas, L., Larroche, C. and Pandey, A. Current Developments in Solid-State Fermentation. (2013) *Biochemical Engineering Journal* **81**: 146-161.

Van Immerseel, F., De Buck, J., Boyen, F., Bohez, L., Pasmans, F., Volf, J., Sevcik, M., Rychlik, I., Haesebrouck, F. and Ducatelle, R. Medium-Chain Fatty Acids Decrease Colonization and Invasion through *Hila* Suppression Shortly after Infection of Chickens with *Salmonella*

Enterica Serovar Enteritidis. (2004a) *Applied and Environmental Microbiology* **70**, 6: 3582-3587.

Van Immerseel, F., De Buck, J., Pasmans, F., Velge, P., Bottreau, E., Fievez, V., Haesebrouck, F. and Ducatelle, R. Invasion of *Salmonella* Enteritidis in Avian Intestinal Epithelial Cells in Vitro Is Influenced by Short-Chain Fatty Acids. (2003) *International Journal of Food Microbiology* **85**, 3: 237-48.

Van Immerseel, F., Fievez, V., de Buck, J., Pasmans, F., Martel, A., Haesebrouck, F. and Ducatelle, R. Microencapsulated Short-Chain Fatty Acids in Feed Modify Colonization and Invasion Early after Infection with *Salmonella* Enteritidis in Young Chickens. (2004b) *Poultry Science* **83**, 1: 69-74.

Van Immerseel, F., Russell, J.B., Flythe, M.D., Gantois, I., Timbermont, L., Pasmans, F., Haesebrouck, F. and Ducatelle, R. The Use of Organic Acids to Combat *Salmonella* in Poultry: A Mechanistic Explanation of the Efficacy. (2006) *Avian Pathology* **35**, 3: 182-8.

Verspreet, J., Damen, B., Broekaert, W.F., Verbeke, K., Delcour, J.A. and Courtin, C.M. A Critical Look at Prebiotics within the Dietary Fiber Concept. (2016) *Annual Review of Food Science and Technology* **7**: 167-90.

Wu, Y.B., Ravindran, V., Pierce, J. and Hendriks, W.H. Influence of Three Phytase Preparations in Broiler Diets Based on Wheat or Corn: *In Vitro* Measurements of Nutrient Release. (2004) *International Journal of Poultry Science* **3**, 7: 450-455.

3

ORAL ADMINISTRATION OF *RUMINOCOCCUS TORQUES* AND *BIFIDOBACTERIUM PSEUDOLONGUM* INCREASES RESILIENCE AGAINST *SALMONELLA* INFECTIONS IN BROILERS

ABSTRACT

Wheat bran is a highly concentrated source of dietary fibres such as arabinoxylans. In a previous study we have shown that wheat bran with reduced particle size can reduce colonization and shedding of *Salmonella* in experimentally infected broilers. This particular wheat bran fraction was enriched with two bacterial species, being *Ruminococcus torques* and *Bifidobacterium pseudolongum*. We hypothesized that the administration of these two strains to *Salmonella* challenged broilers would induce similar effects on colonization and shedding. Therefore *B. pseudolongum*, *R. torques* and a combination of both were grown in liquid broth and broth supplemented with either 1% unmodified wheat bran or 1% wheat bran with a reduced average particle size of 280µm. *B. pseudolongum* and *R. torques* supernatant lowered *Salmonella* invasion gene expression (*hilA*) in the presence of unmodified wheat bran and even more when particle size reduced wheat bran was provided as substrate. The potential of the two species and their combination was evaluated in a *Salmonella* infection model. Both strains reduced the fraction of broilers with high cecal *Salmonella* counts. A synergistic effect could be observed in case of the combination of both strains. Effects of wheat bran with reduced particle size on *Salmonella* colonization can thus be reproduced by using bacterial strains that are associated with this specific substrate.

1. INTRODUCTION

Despite the implementation of control measures, the consumption of contaminated poultry meat is still an important cause of *Salmonella* infections in humans (EFSA 2015). Chickens are often infected at an early age and these infections tend to persist until the age of slaughter which enables the spread into the food chain (Desmidt et al. 1997). Dietary interventions have already proven their worth in the control of *Salmonella* (Van Immerseel et al. 2006, Eeckhaut et al. 2008, Teirlynck et al. 2009, Ravn et al. 2017). Wheat bran is a highly concentrated source of (in)soluble DFs such as cellulose, lignin and AX (Maes & Delcour 2002). We have shown previously that the addition of 1% wheat bran with a reduced particle size of 280 µm (WB280) in poultry feed could significantly reduce cecal colonization and shedding of *Salmonella* in two distinct *Salmonella* infection models (Vermeulen et al. 2017). These results could be at least partly explained by the efficient fermentation of WB280 into SCFA. In a second study we observed that WB280 particles are colonized by a very specific bacterial community (Vermeulen et al., unpublished). Using V3-V4 amplicon sequencing, we could identify two species that were specifically enriched on WB280, being *Bifidobacterium pseudolongum* and *Ruminococcus torques* (Table 1). We hypothesize that the abundance of these two species contributes to the positive results observed in the *Salmonella* infection models. Therefore, *in vitro Salmonella* invasion gene expression was analyzed after incubation of *Salmonella* in medium containing supernatant derived from *B. pseudolongum* and *R. torques* cultures, either or not supplemented with WB or WB280. In addition, the effect of oral administration of both strains, either or not in combination, on *Salmonella* colonization was evaluated in an infection model.

Table 1
Identification of OTUs specifically enriched on wheat bran with reduced particle size. Adapted from (chapter 4)

	ID (% 16S rRNA sequence similarity)	Accession number	Representative strain
OTU2	99% *Bifidobacterium pseudolongum*	LC187663.1	DSM 20099
OTU5	97% *[Ruminococcus] torques*	AB910746.1	ATCC 27756

2. MATERIAL & METHODS

2.1 BACTERIAL STRAINS

Bifidobacterium pseudolongum subspecies *pseudolongum* (DSM 20099) and *Ruminococcus torques* (ATCC 27756) were obtained from the DSMZ and ATCC collections, respectively. The streptomycin resistant *Salmonella enterica* serotype Enteritidis phage type 4 strain 147 (SE147) was used as challenge strain. This strain was originally isolated from egg white and is known to colonize the chicken gut and internal organs to a high extent (Methner et al. 1995).

2.2 MODIFIED WHEAT BRAN FRACTIONS

The particle size of wheat bran was reduced as described previously (Jacobs et al. 2015). In short, commercial wheat bran (Dossche Mills, Deinze, Belgium) was reduced in particle size with a Cyclotec 1093 Sample mill (FOSS, Höganäs, Sweden). By changing the grinding ring and/or the mesh size of the final sieve of the mill, bran fractions with different particle sizes were obtained. The particle size distribution of the resulting fractions was determined by sieving 20.0 g of bran on a set of sieves with mesh sizes of 4500, 2000, 1000, 710, 500, 400, 250, 200, 160, 125, 112, 90, 50, and 38 µm. The set of sieves was shaken for 30 min at a frequency of 1.5 s^{-1} with a retch Vibratory Sieve Shaker (Aartselaar, Belgium) after which each sieve was gently brushed to avoid clogging of the sieve pores. The mass of the bran that remained on each sieve was determined and used to calculate a mass based average particle size:

$d_{av} = \sum d_i \, . \, m_i$

with d_i = (upper size limit of bran fraction i + lower limit of bran fraction i)/2 and m_i = mass fraction, i.e. mass on sieve i/sum of masses of all fractions.

Regular wheat bran (1690 µm) and wheat bran with an average reduced particle size of 280 µm, hereafter referred to as WB and WB280, respectively, were used in the experiments described below.

2.3 *IN VITRO* DIGESTION AND FERMENTATION

The different wheat bran fractions were predigested *in vitro* using a protocol previously described by Wu et al. (2004) which was slightly adapted (Wu et al. 2004). First, 1 g substrate was incubated with 1.5 ml 0.03 M HCl (40°C for 30 min.) to mimic the initial stages of digestion in the crop. Secondly, the digestion in the proventriculus and gizzard was simulated by incubating the substrate with 3000 U of pepsin from porcine gastric mucosa (Sigma-Aldrich, St. Louis, United States) in 1 ml 1.5 M HCl (40°C for 45 min.). To simulate digestion in the duodenum, 1 ml 1 M $NaHCO_3$ and 3.7 mg pancreatin from porcine pancreas (Sigma-Aldrich) were added (2 h at 40°C). Subsequently the predigested bran fractions were centrifuged (5 min., 5000xg) and washed twice with 20 ml of aqua dest. The resulting pellet was retained and lyophilized. The *in vitro* fermentations were performed using a nutrient-poor medium described by Moura et al. (2007) with minor modifications, previously described by De Maesschalck et al. (2015) (Moura et al. 2007, De Maesschalck et al. 2015). The pH of the medium was adjusted to 6.5. The lyophilized predigested fractions were supplemented to the nutrient-poor medium to a concentration of 1% (w/v) and inoculated with $5x10^4$ CFU/ml of either *B. pseudolongum* or *R. torques* or a combination of both strains at a concentration of $2.5x10^4$ CFU/ml. Control conditions did not contain wheat bran. After 24 h anaerobic incubation at 37°C and centrifugation, the supernatant was collected (2500xg, 10 min.).

2.4 MEASUREMENT OF *HILA* EXPRESSION

To determine the expression of the *hilA* gene of SE147, the promotor of *hilA* was cloned in front of *luxCDABE* genes. The production of light is proportional to the expression level of the gene, which was determined relatively to the expression of the housekeeping gene *rpsM*. The construction of a SE147 strain carrying a *hilA*-lux promoter fusion was performed as described previously (Van Immerseel et al. 2004). A *rpsM* promoter fusion was constructed similarly by using *rpsM* specific primers NNNNCTCGAGATCGTTAAGCGTGATGG/ NNNNGGATCCCGACACCGTAGATCGAAG for the initial amplification of the *rpsM* promoter sequence. This resulted in cloning a sequence 214 bp upstream from the *rpsM* start codon containing *rpsM* promoter into pCS26, upstream from the lux reporter genes.

To quantify light production by a SE147 strain carrying plasmids either containing *hilA-luxCDABE* or *rpsM-luxCDABE* transcriptional fusions, a Fluoroscan Ascent fluorometer (Lab-

Systems, Helsinki, Finland) was used. Overnight cultures of SE147 carrying either one of the reporter fusion constructs were diluted 1:100 in Luria Bertani broth (LB, Sigma-Aldrich), supplemented with supernatant (1:1) derived from the different *in vitro* fermentation reactions. As a control, LB (Sigma-Aldrich) was supplemented with supernatant (1:1) derived from the control fermentation without bran. Subsequently the diluted bacterial cultures were grown in microplates at 37°C. Light production was measured automatically every 15 min during 15 hours. The experiment was performed only once.

2.5 ANIMALS

One-day-old Ross 308 broilers were obtained from a local hatchery and housed in containers on wood shavings. A commercial standard wheat based diet and drinking water were provided *ad libitum*. Experiments were performed with the permission of the Ethical Committee of the Faculty of Veterinary Medicine, Ghent University, Belgium (EC 2016_133).

2.6 *IN VIVO* INFECTION TRIAL

To evaluate the effect of the administration of *R. torques*, *B. pseudolongum* and a combination of both strains on the colonization of *Salmonella* in broilers, 120 one-day-old chicks were divided over four treatment groups, each repeated in triplicate (three times ten chickens). The first and second treatment group were administered 10^8 CFU/ml *B. pseudolongum* and *R. torques,* respectively, using oral gavage on day four and five. The third treatment group received a combination of both $5x10^7$ CFU/ml *B. pseudolongum* and $5x10^7$ CFU/ml *R. torques*. The control groups received PBS. At day six, all chickens were challenged with 10^5 CFU of the SE147 challenge strain by oral gavage. Two days post infection (dpi+2), shedding of the SE147 challenge strain was monitored by analysis of cloacal swabs. On dpi+3 the chickens were euthanized and *Salmonella* counts in cecal content were determined. Cloacal swabs were also collected on dpi-1 to ensure that all chickens were *Salmonella* negative prior to experimental infection.

2.7 BACTERIOLOGICAL ANALYSIS

Cloacal swabs were directly inoculated on XLD (Oxoid, Basingstoke, England) plates supplemented with 100 µg/ml streptomycin (Sigma-Aldrich, St. louis, MO, USA). Negative samples were pre-enriched in BPW (Oxoid, Basingstoke, England) overnight at 37°C. One ml

of this suspension was enriched by adding 9 ml of tetrathionate-brilliant green broth (Merck, Darmstadt, Germany). After another overnight incubation at 37°C the suspension was plated onto XLD supplemented with 100 µg/ml streptomycin.

Cecal samples were diluted 10 times and homogenized in BPW, after which 10-fold dilutions were made in PBS. For each dilution, 6 droplets were plated onto XLD plates supplemented with 100 µg/ml streptomycin. These plates were then incubated overnight at 37°C. The number of CFU/g tissue was determined by counting the number of colonies on the plates. Samples negative after direct plating were enriched as described above. Samples negative after direct plating but positive after enrichment were assumed to contain 1×10^1 CFU/g cecum. Samples negative after enrichment were considered to be negative. Bacterial counts were log transformed.

Statistical analysis was performed with SPSS Statistics 23 (IBM Corp, New York, United States), using a Kruskall-Wallis test, followed by a Dunn's post-hoc test to determine statistical differences in the number of *Salmonella* positive cloacal swabs and cecal *Salmonella* counts between groups.

3. RESULTS

3.1 SUPERNATANT DERIVED FROM CULTURES OF *B. PSEUDOLONGUM* AND *R. TORQUES* IN THE PRESENCE OF WB OR WB280 CAN REDUCE *SALMONELLA* INVASION GENE EXPRESSION

B. pseudolongum, *R. torques* and a co-culture of both strains were grown anaerobically in a basal medium (control), basal medium supplemented with 1% WB and 1% WB280 respectively. After 24h, the supernatant was collected. Stationary SE147 cultures carrying a plasmid containing either *hilA*-*lux*CDABE or *rpsM*-*lux*CDABE transcriptional fusions were diluted in LB medium supplemented with supernatant (1:1) from the different fermentations. When *Salmonella* was pretreated with the supernatant derived from the fermentation of *B. pseudolongum* and WB, relatively to the control medium, a reduction in relative *hilA* expression could be observed (Figure 1). In combination with WB280, the effect was even more pronounced. Reductions in *hilA* expression were more subtle when WB and WB280 were provided as substrate for *R. torques*. There appeared to be no synergistic effect when combining both strains since the reduction pattern of the co-culture was similar to that of *R. torques* in all three conditions.

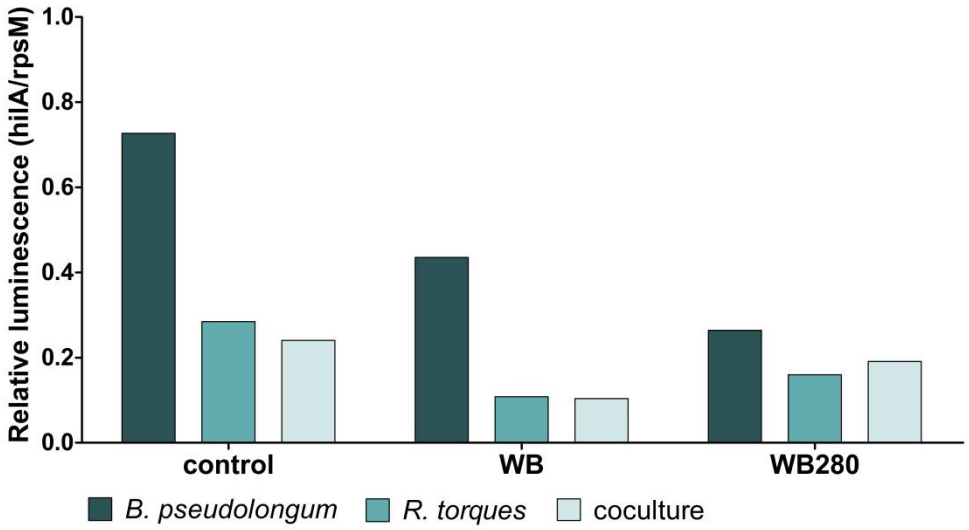


Figure 1 • Relative expression of the *hilA* gene after exposure of *Salmonella* Enteritidis to fermentation products derived from *B. pseudolongum*, *R. torques*, and a combination of both in the absence of wheat bran (control) or in the presence of 1% WB or 1% WB280 (y-axis). Stationary cultures of SE147 carrying a plasmid containing either *hilA-lux*CDABE or *rpsM-lux*CDABE transcriptional fusions were diluted in LB medium supplemented with the supernatants from the respective cultures. The diluted cultures were grown in microplates at 37°C for 15h. The experiment was performed only once.

3.2 *B. PSEUDOLONGUM*, *R. TORQUES* AND A COMBINATION OF BOTH CAN REDUCE CECAL COLONIZATION OF *SALMONELLA* IN A BROILER INFECTION MODEL

Broiler chickens were inoculated with 10^8 CFU/ml of a *B. pseudolongum* or *R. torques* strain or a combination of both. Control animals were inoculated with PBS. Afterwards the animals were challenged with SE147. Before administration of the challenge strain, all animals were *Salmonella* negative. All groups shed the SE147 challenge strain to similar levels, as no statistical differences could be observed between groups (Figure 3). Birds were euthanized and *Salmonella* counts in the cecum were determined at dpi+3. While 53% of the control group chickens showed counts >log6, this was reduced in the group receiving *B. pseudolongum*, *R. torques* or their combination to 20%, 16% ($p<0.05$) and 23% respectively (Figure 2). In accordance, the fraction of birds containing low levels of *Salmonella* in their cecal contents (x<log4) was increased with 10% for *B. pseudolongum*, 17% for *R. torques* and 40% ($p<0.01$) in case of the combined administration compared to the control (Figure 2B). Compared to control animals, the mean log CFU value was reduced with one log unit for birds inoculated with *R. torques* and *B. pseudolongum* and 1.5 log units

with the combined administration of *R. torques* and *B. pseudolongum*. Despite their biological relevance, these results were not statistically significant (Figure 2A).

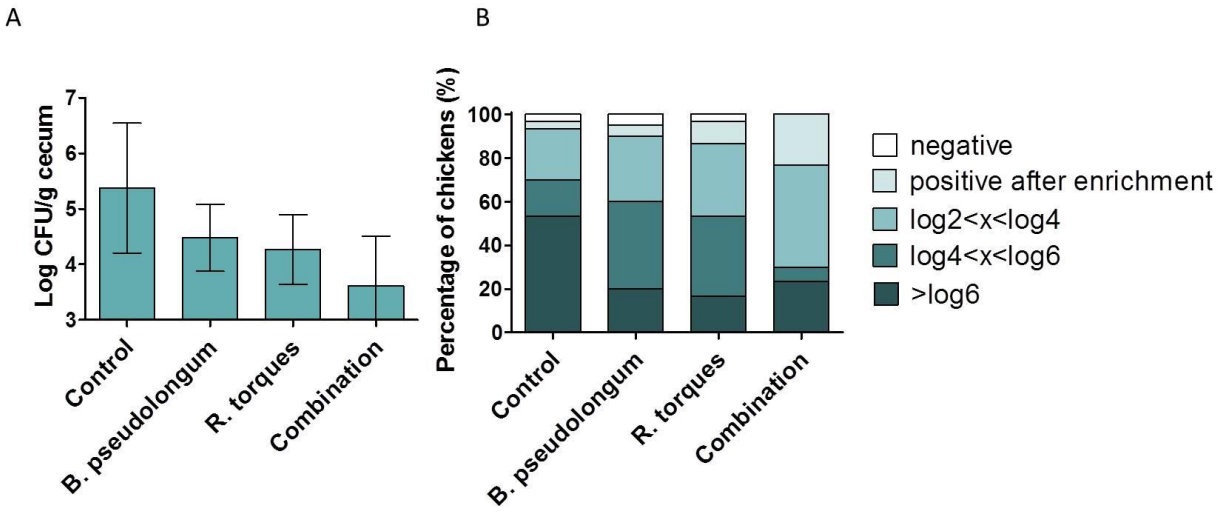
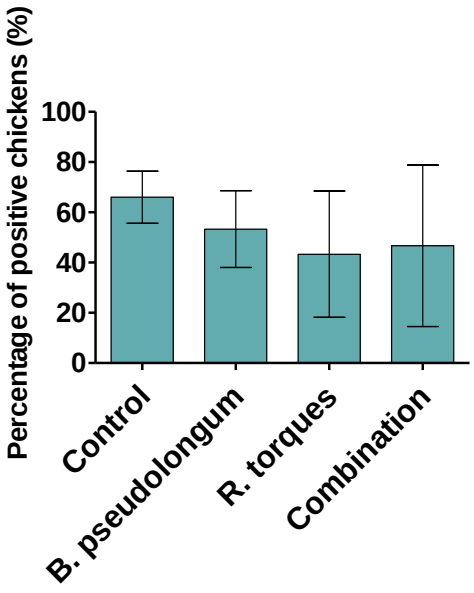


Figure 2 • Cecal colonization by *Salmonella* Enteritidis (SE147). Chickens were inoculated with 10^5 CFU *Salmonella* Enteritidis (SE147) on day 6 of the experiment. At dpi+3, chickens were euthanized and bacterial counts in the ceca were determined. Mean log CFU values and standard deviation per treatment group are shown (A). Percentage of chickens containing a specified log number of CFU per gram cecal content (B).

Figure 3 • Shedding of *Salmonella* strain SE147 after experimental infection of broilers inoculated with *R. torques*, *B. pseudolongum* or a combination of both. Number of positive chickens is shown on dpi+2. Chickens were orally inoculated with 10^5 CFU of *Salmonella* Enteritidis strain SE147 at day 6 of the experiment. Cloacal swabs were collected at dpi-1 and dpi+2. Table shows the number of *Salmonella* shedding animals per group at dpi+2.



4. DISCUSSION

In 2014 in the E.U., the prevalence of broiler flocks positive for *Salmonella* was 3.4%. The most common serovar was *Salmonella* Enteritidis (EFSA 2015). Since the E.U. *Salmonella* control target is defined as a maximum percentage of broiler flocks positive for *S*. Enteritidis or Typhimurium of 1% or less, there is still work to be done. Several approaches using feed additives have already been applied to control *Salmonella* in broilers. Wheat bran derived AXOS, for example, have been shown to reduce shedding and colonization in broilers when administered to the feed (Eeckhaut et al. 2008). Additionally these purified oligosaccharides and soluble AXs have been shown to stimulate beneficial bacteria from the genus *Bifidobacterium* in the gut of broilers (Courtin et al. 2008). In a previous study we demonstrated that WB280 can reduce shedding and cecal colonization rates in broilers infected with *Salmonella* (Vermeulen et al. 2017). Furthermore we observed an enrichment of two specific species on this wheat bran fraction, being *B. pseudolongum* and *R. torques* (Chapter 4). Our hypothesis was that the abundance of these two strains may contribute to the beneficial effects of WB280 supplementation to *Salmonella* challenged broilers.

Current results suggest that both *B. pseudolongum* and *R. torques* release fermentation products that downregulate the expression of the invasion gene *hilA* when WB or WB280 are provided as substrate. The combination of both strains did not seem to have a synergistic effect. At this point the responsible effectors are not yet identified. Most probably the main contributors are specific fermentation products such as SCFA. *R. torques* is a controversial species, known to be associated with inflammatory bowel disease in humans (Hynonen et al. 2016) and with improved performance in broilers (Torok et al. 2013). It belongs to *Clostridium* cluster XIVa, which contains several species that are able to convert lactate into butyrate (Rainey & Janssen 1995, Wilson et al. 1997). Only little information is available on the metabolism of *R. torques* but it is known to encode putative endoxylanases and glucuronidases, both involved in xylan degradation (study 1) (Riviere et al. 2014, Truchado et al. 2015). *B. pseudolongum* can also grow on xylan. Its genome contains sequences encoding putative xylosidases and arabinofuranosidases (study 1). Several mechanisms have been suggested for the inhibitory action of bifidobacteria towards gram-negative pathogens, including a decrease of the local pH by the production of organic acids, competition for nutrients and adhesion sites,

stimulation of the host immune system and the production of specific antibacterial substances (De Vuyst et al. 2004, Servin 2004, Makras & De Vuyst 2006). These antibacterial substances often remain uncharacterized an unidentified (Servin 2004, Makras & De Vuyst 2006).

The observed *in vitro* effects of *R. torques* and *B. pseudolongum* on the expression of *hilA* can be linked with a decrease in cecal colonization *in vivo*. *R. torques* and *B. pseudolongum*, either or not in combination, were administered to broilers that were subsequently infected with *Salmonella*. All three treatments resulted numerically in lower cecal colonization levels compared to the control group of chickens. More specifically, the subpopulation of chickens carrying high numbers of *Salmonella* (> log6) in their cecal content could be statistically reduced when chickens received *B. pseudolongum*, *R. torques* or both, while the subpopulation of chickens carrying low numbers of *Salmonella* increased significantly. These results mirror the observed effects on *hilA* expression, yet, it seems that in the *in vivo* situation the combined administration of the two strains does have a synergistic effect. Whether or not this is due to the orchestrated degradation of wheat bran, the cross-feeding of degradation and/or fermentation products, or the production of compounds with antibacterial properties, has to be investigated.

In conclusion, this work provides a proof of concept. The results obtained by supplementing WB280 to the feed of *Salmonella* challenged broilers could be mimicked by delivering two bacterial species that are enriched on this specific wheat bran fraction. Further research is needed to fully understand the working mechanism. Responsible metabolites which enable the inhibitory effects of *R. torques* and *B. pseudolongum* on *Salmonella* should be identified and characterized.

REFERENCES

Courtin, C.M., Swennen, K., Broekaert, W.F., Swennen, Q., Buyse, J., Decuypere, E., Michiels, C.W., Ketelaere, B.D. and Delcour, J.A. Effects of Dietary Inclusion of Xylooligosaccharides, Arabinoxylooligoscaccharides and Soluble Arabinoxylan on the Microbial Composition of Caecal Contents of Chickens. (2008) *Journal of the Science of Food and Agriculture* **88**, 14: 2517-2522.

De Maesschalck, C., Eeckhaut, V., Maertens, L., De Lange, L., Marchal, L., Nezer, C., De Baere, S., Croubels, S., Daube, G., Dewulf, J., Haesebrouck, F., Ducatelle, R., Taminau, B. and Van Immerseel, F. Effects of Xylo-Oligosaccharides on Broiler Chicken Performance and Microbiota. (2015) *Applied Environmental Microbiology* **81**, 17: 5880-5888.

De Vuyst, L., Avonts, L. and Makras, L. *Probiotics, Prebiotics and Gut Health*, 2004.

Desmidt, M., Ducatelle, R. and Haesebrouck, F. Pathogenesis of *Salmonella* Enteritidis Phage Type Four after Experimental Infection of Young Chickens. (1997) *Veterinary Microbiology* **56**, 1-2: 99-109.

Eeckhaut, V., Van Immerseel, F., Dewulf, J., Pasmans, F., Haesebrouck, F., Ducatelle, R., Courtin, C.M., Delcour, J.A. and Broekaert, W.F. Arabinoxylooligosaccharides from Wheat Bran Inhibit *Salmonella* Colonization in Broiler Chickens. (2008) *Poultry Science* **87**, 11: 2329-34.

EFSA. The 2013 Joint Ecdc/Efsa Report on Trends and Sources of Zoonoses, Zoonotic Agents and Food-Borne Outbreaks Published. (2015) *EuroSurveillance* **20**, 4.

Hynonen, U., Rasinkangas, P., Satokari, R., Paulin, L., de Vos, W.M., Pietila, T.E., Kant, R. and Palva, A. Isolation and Whole Genome Sequencing of a *Ruminococcus*-Like Bacterium, Associated with Irritable Bowel Syndrome. (2016) *Anaerobe* **39**: 60-67.

Jacobs, P.J., Hemdane, S., Dornez, E., Delcour, J.A. and Courtin, C.M. Study of Hydration Properties of Wheat Bran as a Function of Particle Size. (2015) *Food Chemistry* **179**: 296-304.

Maes, C. and Delcour, J.A. Structural Characterisation of Water-Extractable and Water-Unextractable Arabinoxylans in Wheat Bran. (2002) *Journal of Cereal Science* **35**, 3: 315-326.

Makras, L. and De Vuyst, L. The *in Vitro* Inhibition of Gram-Negative Pathogenic Bacteria by Bifidobacteria Is Caused by the Production of Organic Acids. (2006) *International Dairy Journal* **16**, 9: 1049-1057.

Methner, U., Alshabibi, S. and Meyer, H. Experimental Oral Infection of Specific Pathogen-Free Laying Hens and Cocks with *Salmonella* Enteritidis Strains. (1995) *Journal of veterinary medicine. B, Infectious diseases and veterinary public health* **42**, 8: 459-469.

Moura, P., Barata, R., Carvalheiro, F., Girio, F., Loureiro-Dias, M.C. and Esteves, M.P. *In Vitro* Fermentation of Xylo-Oligosaccharides from Corn Cobs Autohydrolysis by *Bifidobacterium* and *Lactobacillus* Strains. (2007) *Food Science and Technology* **40**, 6: 963-972.

Rainey, F.A. and Janssen, P.H. Phylogenetic Analysis by 16s Ribosomal DNA-Sequence Comparison Reveals 2 Unrelated Groups of Species within the Genus *Ruminococcus*. (1995) *Fems Microbiology Letters* **129**, 1: 69-73.

Ravn, J.L., Thogersen, J.C., Eklof, J., Pettersson, D., Ducatelle, R., van Immerseel, F. and Pedersen, N.R. Gh11 Xylanase Increases Prebiotic Oligosaccharides from Wheat Bran Favouring Butyrate-Producing Bacteria in Vitro. (2017) *Animal Feed Science and Technology* **226**: 113-123.

Riviere, A., Moens, F., Selak, M., Maes, D., Weckx, S. and Vuyst, L.D. The Ability of Bifidobacteria to Degrade Arabinoxylan Oligosaccharide Constituents and Derived Oligosaccharides Is Strain Dependent. (2014) *Applied and Environmental Microbiology* **80**, 1: 204-217.

Servin, A.L. Antagonistic Activities of Lactobacilli and Bifidobacteria against Microbial Pathogens. (2004) *FEMS Microbiology Reviews* **28**, 4: 405-440.

Teirlynck, E., Haesebrouck, F., Pasmans, F., Dewulf, J., Ducatelle, R. and Van Immerseel, F. The Cereal Type in Feed Influences *Salmonella* Enteritidis Colonization in Broilers. (2009) *Poultry Science* **88**, 10: 2108-2112.

Torok, V.A., Dyson, C., McKay, A. and Ophel-Keller, K. Quantitative Molecular Assays for Evaluating Changes in Broiler Gut Microbiota Linked with Diet and Performance. (2013) *Animal Production Science* **53**, 12: 1260-1268.

Truchado, P., Van den Abbeele, P., Riviere, A., Possemiers, S., De Vuyst, L. and Van de Wiele, T. *Bifidobacterium Longum* D2 Enhances Microbial Degradation of Long-Chain Arabinoxylans in an in Vitro Model of the Proximal Colon. (2015) *Beneficial Microbes* **6**, 6: 849-860.

Van Immerseel, F., De Buck, J., Boyen, F., Bohez, L., Pasmans, F., Volf, J., Sevcik, M., Rychlik, I., Haesebrouck, F. and Ducatelle, R. Medium-Chain Fatty Acids Decrease Colonization and Invasion through *Hila* Suppression Shortly after Infection of Chickens with *Salmonella Enterica* Serovar Enteritidis. (2004) *Applied and Environmental Microbiology* **70**, 6: 3582-3587.

Van Immerseel, F., Russell, J.B., Flythe, M.D., Gantois, I., Timbermont, L., Pasmans, F., Haesebrouck, F. and Ducatelle, R. The Use of Organic Acids to Combat *Salmonella* in Poultry: A Mechanistic Explanation of the Efficacy. (2006) *Avian Pathology* **35**, 3: 182-8.

Vermeulen, K., Verspreet, J., Courtin, C.M., Haesebrouck, F., Ducatelle, R. and Van Immerseel, F. Reduced Particle Size Wheat Bran Is Butyrogenic and Lowers *Salmonella* Colonization When Added to Poultry Feed. (2017) *Veterinary Microbiology* **198**: 64-71.

Wilson, K.H., Ikeda, J.S. and Blitchington, R.B. Phylogenetic Placement of Community Members of Human Colonic Biota. (1997) *Clinical Infectious Diseases* **25**: S114-S116.

Wu, Y.B., Ravindran, V., Pierce, J. and Hendriks, W.H. Influence of Three Phytase Preparations in Broiler Diets Based on Wheat or Corn: *In Vitro* Measurements of Nutrient Release. (2004) *International Journal of Poultry Science* **3**, 7: 450-455.

PART 4

GENERAL DISCUSSION

PARTICLE SIZE REDUCTIONS: NOT AS STRAIGHTFORWARD AS IT MAY SEEM

As stated in the introduction, wheat bran is a very rich source of DF, so therefore it is worthwhile to study the effects of wheat bran supplementation on intestinal health. Moreover, it was made clear that technical modification can change the physicochemical properties of DF and potentially improve its functionality (Dhingra et al. 2012, Jacobs et al. 2015b, Jacobs et al. 2016b, Vermeulen et al. 2017). These processes need to be thoroughly characterized even for modifications 'as simple' as particle size reductions.

1. REDUCTION AND CHARACTERIZATION

Particle size reduced wheat bran fractions used in this work were obtained by a Cyclotec 1093 Sample mill (Jacobs et al. 2015b). The reduction of particle size depends on the joint action of the rotor, the grinding ring, and the mesh size of the sieve through which the sample has to pass (Jacobs et al. 2015b). The resulting particles are appointed an average size but in reality they represent a range of particle sizes (Figure 1). When using different equipment or alternative protocols during the milling process, this size distribution can differ significantly. Industrially, cereal grains are milled mostly by means of either a hammer mill or roller mill. These two milling systems each generate particles with different characteristics and size distributions. It has been shown that roller mills generate more irregularly shaped particles with a relative uniform size distribution, while hammer milling results in more spherical particles with a less uniform size distribution (Amerah et al. 2007). Thus, it is of uttermost importance to characterize these distributions (Kalivoda et al. 2017). Both the tools and techniques used to characterize bran fractions are crucial in determining the outcome. Depending on the size and nature of the substrate, certain techniques are more appropriate than others and often the most appropriate approach is to use a combination of several methods (Hrncırova et al. 2013). The relatively simple sieving method is based on the utilization of a set of sieves with known mesh size. The particle size distribution is calculated by measuring the mass of particles retained on each sieve. In general, particles from 4 mm down to 40 µm can be analyzed using this method. Laser diffraction uses a laser light which is scattered by a particle in suspension when passing through the laser beam. Based on the obtained light distribution, the size distribution of the particles can be calculated. This method is

frequently used to determine the size of small particles ranging from 0.08 µm to 2 mm (Hrncirova et al. 2013). All of these technical aspects need to be taken into account when investigating the effects of particle size on fermentability. It is a warrant against simple extrapolation of the results of the present studies.

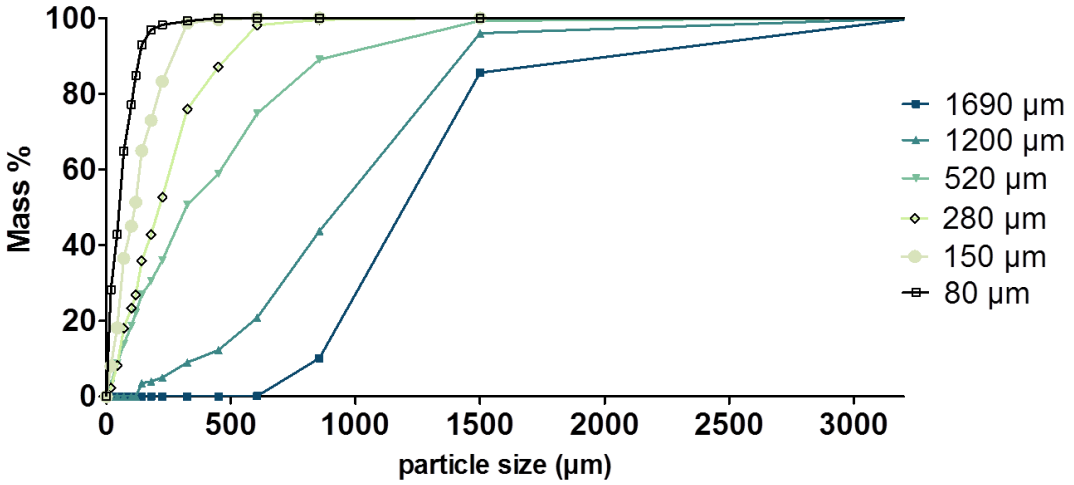


Figure 1 • Mass based cumulative particle size distributions of wheat bran before (1690 µm) and after milling. Legend shows average reduced particle size of the different samples. Size distributions were determined once only. Adapted from Jacobs et al. (2015).

2. FERMENTABILITY & COLONIZATION PATTERNS

When administering wheat bran with reduced particle size to *Salmonella* infected broilers, a fraction with average reduced particle size of 280 µm was identified which suppressed cecal colonization and shedding. This effect could not be achieved to the same extent when feeding particles with larger or smaller average particle sizes. Apparently there is some kind of optimum. One can expect that this is rather due to an optimal particle size distribution than an actual optimal average particle size. There are indications that plant cell wall constituents presented as fine particles are more available for digestive enzymes and bacterial fermentation due to the increased surface area (Stewart & Slavin 2009, Jacobs et al. 2016a). Our data confirm this statement in the sense that, when comparing SCFA production from coarse (1690 µm) and fine (280 µm) wheat bran particles, a significantly higher total amount of SCFA was produced from fine particles. The *Salmonella* infection trials indicated that the effect of wheat bran with even smaller average particle sizes (150 µm and 80 µm) could provide some kind of resilience to

Salmonella since numerically the fraction of *Salmonella* negative broilers in the respective groups was lower than that of the control group or groups receiving coarser wheat bran particles. Yet, the effect was not as clear as for broilers receiving WB280. We can only guess what the underlying mechanism could possibly be. One possibility is that, due to particle size reduction, the structure and availability of the bran constituents change. As shown by Jacobs et al. (2015), the chemical composition of wheat bran does not change upon particle size reduction but the extractability of AX and β-glucan does increase, just like the damaged starch content (Jacobs et al. 2015a). This increased accessibility of hemicelluloses may initiate the fermentation process higher up in the gastrointestinal tract, reducing the available substrate for cecal fermentation. In order to efficiently affect *Salmonella* colonization in the ceca, SCFA should be produced *in situ*, in the ceca.

Besides, there are strong indications that the colonizing bacterial community could be affected as well. We have shown that WB280 is colonized by a very distinct bacterial community compared to regular wheat bran (WB) particles. If wheat bran particles smaller than WB280 are already (partly) fermented in the upper gastrointestinal tract, their chemical composition changes, and subsequently they may attract a different bacterial community which may not be enriched in members of the *Lachnospiraceae* family as is the case for WB280. At this point we cannot back this hypothesis with data since we did not investigate the bacterial community specifically colonizing the smaller wheat bran fractions. This is something that may be worth looking into in the future.

3. Mechanical aspects associated with particle size

Not only does particle size seem to affect fermentation rate, it is likely to induce differential mechanical effects as well. Larger particles are known to stimulate the development of the GIT and gizzard (Jimenez-Moreno et al. 2010, Svihus 2014). In addition, larger particles are retained longer in the gizzard compared to smaller ones. Since functionality of the digestive tract will possibly have a large effect on response to dietary manipulations (e.g., enzyme and pre- or probiotics addition), it needs to be taken into account (Svihus 2014). Svihus (2011) formulated a recommendation regarding particle size of poultry diets: feeds should include at least 30% particles larger than 1 mm in size to

stimulate gizzard development (Svihus et al. 2004b, Svihus 2011). This statement was supported by a correlation analysis of particle size and gizzard size made for ten different diets (Svihus et al. 2004b). Secondary effects of a well-developed gizzard and GIT are reduced gizzard pH and increased bile and enzyme secretions (Hetland et al. 2003, Svihus et al. 2004a). All these factors favor efficient absorption of nutrients. In addition, higher gizzard acidity reduces pathogen colonization.

Smaller particles do not tend to stimulate gizzard development, are retained in the gizzard for shorter periods and tend to move faster through the gastrointestinal tract (Hetland et al. 2004). A fast transit time, averts efficient digestion and absorption of nutrients and water. In addition, larger wheat bran particles, have been shown to possess a larger water holding and swelling capacity than smaller particles. (Jacobs et al. 2015a). Bound water will lead to less problems with wet litter and associated conditions such as footpath lesions.

The commercial diet to which WB280 was supplemented in the *in vivo* experiments was measured to have an average particle size of 885±101 µm with 30% of the particles being larger than 1 mm. So one can suspect that the addition of 1% WB280 did not cause shifts in the particle size distribution of the basal feed and thus would not have impaired GIT development and/or activity.

THE ROLE OF THE BASAL DIET AND CHICKEN STRAIN

For our experiments we used Ross 308 broilers fed with a wheat/rye-based mash diet (Table 1). In Belgium and Europe, this is the most frequently used broiler breed.

As was discussed throughout this thesis, the microbiota influences both the immune and nutritional status of birds but the host in its turn can mold the microbiota composition as well. Three host-related aspects can have significant influence on the intestinal microbiota composition:

- digestive physiology (turnover intestinal epithelium, quantity of mucus, motility of the gut, the nature and amount of intestinal secretion),
- composition of the intestinal content (depends on the digestive efficiency of the bird and the composition of the diet) and,

- the presence of antimicrobial compounds generated by immune and epithelial cells.

All three factors are influenced by host genetics and host genotype and have been shown to indirectly influence the microbiome through modification of its biotope (Zhao et al. 2013). This principle was elegantly illustrated by Mignon-Grasteau and coworkers. After eight generations of selection, the authors obtained two broiler lines which differed up to 40% in digestive efficiency (Mignon-Grasteau et al. 2015). These two lines of 'good' and 'bad' digesters exhibited, in addition to differences in intestinal microbiota composition, as well as differences in acid and bile acid secretions (Tran et al. 2014), gut motility (Rougiere et al. 2012) and structure of the intestinal epithelium (de Verdal et al. 2010). Rougière and others (2012) observed that the 'good' digesters, for example, showed a slower transit time. The slower transit time promotes the development of bacterial species able to survive in harsh conditions, whereas the development of bacteria able to proliferate fast is favored in birds with a high transit rate (Rougiere et al. 2012). This clearly illustrates that the genetic background of the host can influence on the microbiota composition of the gut. This implicates that the addition of WB280 is expected to result in different shifts in microbiota composition in different broiler strains since the basal composition is shaped by the prevailing circumstances in the gut.

Next to host genetics, the basal diet can influence the outcome of WB280 supplementation as well. The grain type, and especially DF content and the nature of the DF present in the grain could influence the outcome of WB280 supplementation. Indeed, Knudsen (2014) has shown that DF content varies greatly for different crops; for example, β-glucan, cellulose and AX content was shown to be significantly different for corn, wheat and rye grains (Knudsen 2014). Also, the average particle size (see above) of the feed and formulation (mash versus pellets) could affect the results. Another important factor is the presence of enzyme preparations. By supplementing feed with xylanases or phytases for example, one increases digestibility by degradation of the AX structures (Cowieson 2010). Considering the fact that wheat bran contains high amounts of AX, the addition of these kind of enzymes could significantly alter the outcome of in-feed WB280 supplementation. Thus, depending on the basal diet, the effects that we observe could be obscured or even enhanced when added to a different basal diet. This means

that, for the time being, the conclusions drawn from the present studies should be restricted to the conditions of the present experiments. Further studies using different strains of birds and using different feed formulations can reveal whether the conclusions from the present studies can be extrapolated to other conditions.

In the future, it would be interesting to investigate the effects of WB280 supplementation on the microbiota composition in different broilers breeds such as Cobb 500, slow growing broilers (e.g. Sasso), and perhaps even in layers. By changing the basal diet and its formulation, an optimal combination of diet and supplement may be identified.

Table 1
Nutrient composition of the commercial wheat/rye-based mash feed used in the broiler experiments in this thesis.

Nutrient composition	
Crude protein	21%
Crude fat	6%
Crude ash	5.5%
Crude fibre	3%
Lysine	1.17%
Methionine	0.52%
Calcium	0.85%
Phosphorous	0.58%
Sodium	0.15%

CAN WHEAT BRAN BE CONSIDERED A PREBIOTIC?

A disturbed gut microbiota is often characterized by reduced numbers of beneficial bacteria and increased proliferation of harmful (pathogenic) bacteria. In broilers it can predispose for infections with e.g. *Salmonella* (Rivera-Chavez et al. 2016) or/and it can cause poor performance (Torok et al. 2013). It is well known that the microbiota composition of the gut can be influenced by dietary interventions. A **prebiotic** is defined as a selectively fermented dietary ingredient that results in specific changes in the composition and/or activity of the gastrointestinal microbiota, conferring benefit(s) upon host health (Gibson et al. 2004a, Roberfroid et al. 2010). Benefits of prebiotics include improvement of gut barrier function and host immunity, reduction of potentially pathogenic bacteria and the enhancement of SCFA production (Slavin 2013). Following the definition of a prebiotic, all

prebiotics are DFs but not all DFs are prebiotics (Slavin 2013, Verspreet et al. 2016). For DF to be classified as prebiotic, three criteria must be met: the ingredient should

(i) resist gastric acidity, hydrolysis by host enzymes and gastrointestinal absorption,
(ii) be fermented by the intestinal microbiota,
(iii) stimulate selectively the growth and/or activity of intestinal bacteria associated with health and wellbeing (Gibson et al. 2004b).

Wheat bran is a highly concentrated source of (in)soluble DF which, according to our results, meets all three of the above mentioned criteria.

(i) The main constituent of wheat bran is AX, recognized as DF, meaning it will reach the hindgut undigested.
(ii) Using batch fermentations we demonstrated that it is efficiently fermented by the complex cecal microbiota of broilers in butyrate, propionate and acetate.
(iii) Its potential as feed additive to improve gastro-intestinal health of broilers was investigated. The supplementation of only 1% WB280 to the feed of broilers could alter the Firmicutes to Proteobacteria ratio by stimulating butyrate producers from the *Ruminococcaceae* and *Lachnospiraceae* families and reducing members of the *Enterobacteriaceae* in the ceca. Moreover, *in vitro* it was demonstrated that wheat bran was specifically colonized by species potentially exerting xylan degrading activities. The community attached to WB280 was enriched in bifidobacteria and members of the *Lachnospiraceae* family.

These results suggest that wheat bran with reduced particle size could be an interesting alternative to the purified oligosaccharides that are already commonly used.

COULD WHEAT BRAN BE PART OF A SYNBIOTIC FORMULA?

Probiotics are also frequently applied to restore a healthy gut microbiota or to increase broiler performance. A **probiotic** is a live microorganism that, when administered in adequate amounts, confers a health benefit on the host (Hill et al. 2014). Probiotics are able to induce both compositional as functional changes in the gut microbiota (De Preter et al. 2011). The most frequently applied probiotic strains belong to the genera *Lactobacillus* and *Bifidobacterium* *(Wilkins & Sequoia 2017)*. In this thesis we identified several bacteria that

were specifically attached to WB280, being *B. pseudolongum* and *R. torques* (study 1). These species could be potential probiotic candidates since they were able to numerically reduce *Salmonella* colonization in the ceca when administered by oral gavage (study 3). In contrast to prebiotics, working with probiotics is somewhat challenging because they need to survive the acidic environment of the stomach and reach the location in the intestine where they are most useful. In this specific case it might be more interesting to deliver the appropriate substrate, here being WB280, together with the colonizing, beneficial strain(s) and thus consider the principle of a synbiotic. **Synbiotics** are the combination of prebiotic substrates and compatible probiotic strains (Schrezenmeir & de Vrese 2001). The administration of both components often results in a synergistic effect (Gallaher & Khil 1999). Since wheat bran particles are characterized by an irregular porous structure, bacteria have the opportunity to reside in a protective environment. In study 1, we identified OTUs that are specifically enriched on WB280. Among these sequences, several OTUs could be appointed to lactate producing bifidobacteria and lactate consuming, butyrate producing *Lachnospiraceae*. The combined administration of both functional groups on their preferred substrate may enable them to interact efficiently and may confer significant gastrointestinal health effects. The main advantage of this approach is that the substrate is immediately colonized by the desired microbial community.

POTENTIAL MECHANISMS THAT ENABLE COLONIZATION RESISTANCE AGAINST *SALMONELLA*

As already mentioned in the general introduction, commensal bacteria play a crucial role in the defense against pathogen invasion. Several strategies providing colonization resistance can be distinguished, including: metabolic exclusion by production of bacteriocins and SCFA, competition for nutrients and niches, and immune-mediated mechanisms (both innate and adaptive). We observed a clear enrichment of the genera *Lactobacillus*, *Bifidobacterium*, *Enterococcus*, *Bacteroides* and butyrate-producing genera from the *Lachnospiraceae* and *Ruminococcaceae* families on bran particles. Several species within these genera have been proven to contribute to colonization resistance in one way or another. Bellow some examples are described for each of them.

Lactobacillus

Kim et al. (2015) isolated several *Lactobacillus* strains from broilers that showed bacteriocin-like activity against several *Salmonella* species. The isolates showed high sequence similarity with *Lactobacillus crispatus* and *Lactobacillus johnsonii* (Kim et al. 2015). A *Lactobacillus salivarius* isolate, obtained from broilers, showed bacteriocin activity as well: bacteriocin L-1077 proved to protect against *Campylobacter* and *Salmonella* both *in vitro* and *in vivo* (Svetoch et al. 2011).

Commensals like *Lactbacillus rhamnosus* employ a different strategy. This species was shown to outcompete pathogens like *Salmonella* for attachment sites by means of mucus-binding pili (Kankainen et al. 2009).

Bifidobacterium

Makras & De Vuyst (2005) screened several bifidobacteria for their inhibitory activity against indicator organisms by means of bacteriocin production. Some of them affected gram positive bacteria and *E. coli* but the effects on other gram negative bacteria such as *Salmonella* were found to be negligible (Makras & De Vuyst 2006). However, they observed another mechanism employed by bifidobacteria which did confer inhibition of *S*. Typimurium *in vitro*. Investigation of the inhibitory mechanism revealed that the activity was dependent on the production of organic acids, in particular acetate and lactate, and the lowering of the pH of the medium (Makras & De Vuyst 2006). It is unclear whether these mechanisms also play a role *in vivo*.

Enterococcus

Enterococcus species are also known for their bacteriocin activity. *E. faecalis* and *E. faecium* are both inhabitants of the chicken GIT and haven proven to be effective in *Salmonella* inhibition by means of bacteriocins *in vitro* (Kang & Lee 2005, Perumal et al. 2016).

Bacteroides

The intestinal commensal bacterium *Bacteroides thetaiotaomicron* antagonizes intestinal pathogens through a range of mechanisms that include the activation of host immune defences and direct interactions with other intestinal bacteria (Buffie & Pamer 2013).

For example, *B. thetaiotaomicron* colonization in mice enhanced the expression of the antimicrobial C-type lectins REGIIIγ and REGIIIβ in the large intestine (Sonnenburg et al. 2006). These and other host-derived antimicrobials are concentrated in the mucus layer and make it even less permeable for pathogens (Meyer-Hoffert et al. 2008).

Salmonella uses fimbriae and pili to adhere to a wide range of targets. The *Salmonella std* operon encodes a fimbrial adhesin which binds α-1-2 fucosylated receptor molecules in the mucus layer. Using fucosidases, commensals like *B. thetaiotaomicron* cleave off fucoses, dismantling the attachment sites (Ng et al. 2013).

Lachnospiraceae and *Ruminococcaceae*

Not only did we observe an increase in relative abundances of butyrate producing *Ruminococcaceae* and *Lachnospiraceae*, we could also show that the fermentation of WB280 to SCFA was much more efficient than for regular wheat bran, both leading to increased SCFA levels. In high concentrations, SCFA such as butyrate, propionate and acetate, can inhibit pathogens such as *E. coli* (Cherrington et al. 1991, Shin et al. 2002), *C. difficile* (Rolfe 1984) and *S.* Typhimurium (Bohnhoff et al. 1964, Van Immerseel et al. 2006b) at least *in vitro*. As was already elaborately discussed, SCFA can affect *Salmonella* virulence gene expression as well (Gantois et al. 2006, Hung et al. 2013).

Salmonella exploits the immune system of the host to invade and spread systemically (Behnsen et al. 2015). Moreover, it has been shown that *S*. Typhimurium can alter the microbiota composition by promoting intestinal inflammation (Stecher et al. 2007). The bacteria attract phagocytic cells and are capable of replicating within these cells (Behnsen et al. 2015). SCFA, and especially butyrate, are important regulators of the immune system. For example, C2, C3 and C4 SCFA have been shown to steer the differentiation of T cells into Tregs and induce IgA production by B cells (Kim et al. 2016). Moreover, butyrate can suppress inflammation by regulating the expression of NFκB (Inan et al. 2000). A cocktail of rationally selected *Clostridium* species was shown to induce regulatory T cell development (Atarashi et al. 2011). Another mechanism relies on the inhibition of histon deacetylaes which in its turn increases histone H3 acetylation in the Foxp3 promoter, a master regulator of Treg development (Furusawa et al. 2013). By inducing Tregs, inflammation is tempered and systemic spread can be impaired. Moreover, acetatate, propionate and

butyrate have been shown to induce TGF-β expression by intestinal epithelial cells which led to Treg development as well (Atarashi et al. 2013).

The aforementioned examples illustrate how the bran-induced enrichment of specific genera and species could explain the reductions in relative numbers of *Enterobacteriaceae* and reduction in *Salmonella* colonization rates in the infections trials we observed.

CAN WB280 CONFER PROTECTION TO OTHER IMPORTANT BROILER PATHOGENS?

As stated above, pre-, pro- and synbiotics confer health benefits by steering activities and/or composition of the gut microbiota. This includes increasing the resilience against pathogens. In industrial poultry production, eggs are separated from the parental flocks prior to hatching. This reduces the parental influence on the microbiota composition (Stanley et al. 2014). The microbiota, as well as the immune system of young birds, is therefore often underdeveloped. Moreover, during the rearing period, birds are placed in confined, stressful environments. All these factors make the birds highly susceptible to various bacterial infections.

The supplementation of WB280 to *Salmonella* infected broilers could reduce both colonization and shedding. We postulated that this was due to the more efficient fermentation of the smaller wheat bran particles and the resulting increased concentration of butyrate and propionate (study 2). These SCFA have the ability to downregulate the expression of several invasion related genes, resulting in a lowered invasion potential of *Salmonella* and increased resilience of broilers against *Salmonella* infections (Gantois et al. 2006, Van Immerseel et al. 2006a). Butyrate is a signaling molecule in the lower gastrointestinal tract which appears to be sensed by many different microorganisms as well as by the host. Therefore one can speculate that butyrate may affect other pathogens as well.

1. *CLOSTRIDIUM PERFRINGENS*

Necrotic enteritis (NE) is a multifactorial gastrointestinal disease in both layers and broilers. The causative agent is *Clostridium perfringens* but for this bacterium to cause

intestinal lesions, several predisposing factors need to be present, being a suppressed immunne system, a feed high in animal proteins and NSP levels and coccidiosis inflicted lesions. Eeckhaut et al. (2016) observed that the administration of the butyrate producing *Butyricicoccus pullicaecorum* strain 25-3^T could contribute to the prevention of NE (Eeckhaut et al. 2016). Butyrate as such in combination with essential oils and medium chain fatty acids have proven their value in a NE model as well (Timbermont et al. 2010). However, our unpublished results suggest that the administration of 1% WB280 is not efficient in reducing the number of birds with lesions. The mean total lesion scores were not different for control chickens or chickens receiving WB or WB280 but a small increase in birds with low total lesion scores could be observed in the latter two groups (Figure 2). Most probably the induced lesions are too severe to prevent or control by means of any prebiotic compound. Butyrate levels obtained from WB280 fermentation may be insufficiently high to prevent lesions or induce a healing effect.

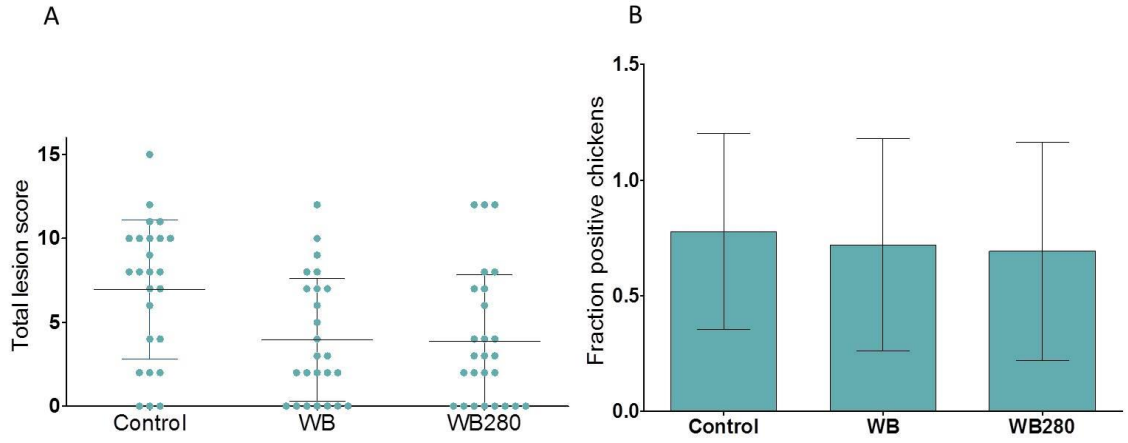


Figure 2 • The effect of the supplementation of 1% WB and WB280 in a subclinical necrotic enteritis model. The immune system of the birds was suppressed by vaccination with Gumboro vaccine. Small intestinal lesions were inflicted by administering of a 10-fold dose of a commercial coccidiosis vaccine. The feed was high in NSPs and during finisher period fishmeal is used as source of animal protein. During the last days of the trial, chickens are inoculated on three consecutive days with 10^8 CFU/ml of a *C. perfringens* strain. A control group received unsupplemented feed while a second and third group received feed with 1% of WB and WB280 respectively. The percentage of lesion negative chickens per group was determined (B), as well as the total lesion score per chicken per group (A). Mean and SD are shown.

2. *CAMPYLOBACTER JEJUNI*

Campylobacter jejuni is the lead species causing *Campylobacter* enteritis in humans (EFSA 2015). Like *Salmonella* it is a zoonotic agent which is spread through the consumption of contaminated meat products (Hermans et al. 2012). *Campylobacter* has the preference of colonizing the lower intestinal tract, and more specifically the ceca of broilers (Jacobsreitsma et al. 1995, Evans & Sayers 2000). No effective strategy exists to clear *Campylobacter* from broiler flocks (Hermans et al. 2011). An *in vitro* study showed that butyrate is able to protect Caco-2 cells by reducing the invasion and translocation potential of *C. jejuni* (Van Deun et al. 2008b). Despite the marked bactericidal effect of butyrate towards *C. jejuni in vitro*, butyrate-coated micro-beads could not protect broilers from cecal colonization as was demonstrated in an *in vivo* study by the same group (Van Deun et al. 2008a). Moreover, Luethy et al. (2017) found that *C. jejuni* responds to physiological concentrations of butyrate by increasing the expression of genes required for growth and survival. Lactate, which is abundant in the upper gastrointestinal tract where *C. jejuni* colonizes less efficiently, decreased the expression of the genes under study. Spatial gradients within the gut seem to determine the tropism for the lower part of the intestine where butyrate provides the signal for proliferation (Luethy et al. 2017). These data suggest that stimulation of butyrate production by supplementing WB280 will probably not inhibit *Campylobacter* growth and/or colonization.

3. *ENTEROCOCCUS CECORUM*

The translocation of pathogens over the intestinal barrier enables their systemic spread. *Enterococcus cecorum* is an enteric commensal which can cause so called enterococcal spondylitis in broilers upon translocation (Borst et al. 2016, Wideman 2016). Factors that enhance intestinal integrity are effective in preventing bacterial translocation. Tight junctions are a key component of this intestinal barrier by sealing the apical surface of adjacent epithelial cells (Matter et al. 2005). As stated before, butyrate has the ability to reinforce the gastrointestinal barrier for example through the upregulation of tight junction proteins (Wang et al. 2012). In this case, the beneficial effects are directed to the host and not so much directly to the pathogen. Since WB280 has been shown to stimulate butyrate production and/or proliferation and activity of butyrate producers, a hypothetical

increased resilience against bacterial translocation of (opportunistic) pathogens such as *E. cecorum* by reinforcing indirectly the intestinal barrier can be expected. This, however, should be investigated further.

CAN WB280 BE APPLIED TO IMPROVE GASTROINTESTINAL HEALTH IN HUMANS?

Since this work has shown that WB280 can induce microbiota shifts in the ceca of broilers which are deemed to be beneficial, it would be plausible to assume that a relatively stable microbiota composition can be installed with accompanying increased SCFA production by the intake of WB280 in humans.

The fact that the microbial community of the gut is strongly influenced by diet applies to both humans and animals (Ley et al. 2006, Middelbos et al. 2010). Like the anatomy of the alimentary tract, the gut microbiome is co-evolved to meet the physiological needs of both chickens and humans (Lei et al. 2012). Despite the fact that their anatomy and physiology differ, two important bacterial families, *Lachnospiraceae* and *Ruminococcaceae*, seem to dominate their microbiome (Jozefiak et al. 2010, Lozupone et al. 2012). Moreover, Lei et al. (2012) showed that several genera, i.e. *Bacteroides*, *Roseburia*, *Faecalibacterium* and *Parabacteroides*, occur in chickens and in humans while at the species level the composition is more divergent.

However, the intestinal tract of humans and chickens is anatomically very different. The alimentary tract of chickens harbors several specific compartments which are adapted to their living conditions: crop, proventriculus or glandular stomach, the ventriculus or gizzard and two cecal pouches. The ceca are the most important site of fermentation in broilers. For humans, the primary site of fermentation is the colon (Windey et al. 2012). The entrance to the ceca of chickens is narrow and lined with villi, filtering out larger insoluble particles while in humans all insoluble particles pass through the colon (Lai & Duke 1978, Clench 1999). However, our own unpublished results indicate that the upper particle size limit of this sieve mechanism is rather high, allowing particles at least up to 750 µm to the ceca of chickens. Since we did not include particles larger than 750 µm in our experiment, we cannot define the upper limit of particle size. The anatomical

differences between humans and chickens will affect the availability and fermentability of dietary fibres, promoting a different microbiota composition.

Another important factor discriminating humans from chickens is the fact that both variation in diet and genetic variation among chickens from the same breed is small or even non-existent. This is in sheer contrast with the human situation where differences in diet and genetic background are important. Nonetheless, De Paepe et al. (2017) were able to observe a general trend upon wheat bran fermentation when using a fecal inoculum from ten different human donors. Batch fermentations inoculated with fecal material were used to characterize the wheat bran-attached and planktonic bacterial communities. While genus level shifts were characterized by donor variability, the enrichment of *Lachnospiraceae* on the bran was consistent in all donors (De Paepe et al. 2017).

This could implicate that general observed trends may be extrapolated from animals to humans despite of the interspecies variation and despite of the large interindividual differences that typify humans.

MECHANISMS OF BACTERIAL ATTACHMENT

Attachment to insoluble dietary fibres prevents bacteria from washout. In addition, the specific attachment to dietary platforms presumably increases the efficiency of harvest and distribution of oligo- and monosaccharide among the members of the adhered community and beyond (Backhed et al. 2005). Bacterial attachment to (a)biotic surface is mediated by means of extracellular adhesive organelles including pili (or fimbrae), curli and/or flagellae (Van Houdt & Michiels 2005). Xylanolytic *Bacteroides* species are known to employ xylan-utilization (XUS) systems, which are membrane associated enzyme complexes involved in the extracellular binding and transport of xylan fragments (Dodd et al. 2011, McNulty et al. 2013, Rogowski et al. 2016). The mechanism of enzyme mediated attachment has been studied extensively in *Ruminococcus* species (Ezer et al. 2008, Morais et al. 2016). *Ruminococcus albus* binds to and degrades crystalline cellulosic substrates via an unique cellulose degradation system. A unique family of carbohydrate-binding modules, located at the C terminus of different glycoside hydrolases, appears to be responsible both for anchoring these enzymes to the bacterial cell surface and for substrate binding (Morais et

al. 2016). It would be worthwhile to elucidate the attachment mechanisms employed by the taxa specifically enriched on WB280 to understand why these organisms turn to this specific substrate. Understanding the preferences of specific bacteria, could be very helpful when trying to modify DFs as such that attachment is promoted.

THE DINNER TABLE PRINCIPLE

Bäckhed et al. (2005) have postulated that particulate nutrient scaffolds (e.g mucus, food particles and/or exfoliated epithelial cells) can function as 'dinner table' which bacteria can use for both attachment and as nutrient source. When primary degraders and their cross feeding partners can abide in close proximity, this will result in an efficient transfer of degradation products (Macfarlane 2000, Flint et al. 2008, Walker et al. 2008). As stated in the introduction, wheat bran is composed of both fermentable and less fermentable constituents, the latter providing the table while the former provide the dinner. One can wonder if it would be sufficient to provide solely a table, or inert substrate for bacteria to attach and interact without the dinner and whether this would be efficient in promoting beneficial effects in the intestine. Our own unpublished results suggest that this would not be the case. When administering a destarched, pericarp enriched (DSPE) wheat bran fraction to the feed of *Salmonella* infected broilers, shedding and cecal colonization rates could not be reduced compared to a positive control group receiving unsupplemented feed. This DSPE fraction was obtained by the sequential removal of starch, using amylases, and aleurone, using xylanases. All water extractable compounds (e.g. β-glucan, fructan, AXOS, glucose…) were afterwards removed by washing. What remains is relatively inert substrate which is difficult to ferment. Our results indicate that some form of fermentable fibre is needed to obtain beneficial effects, at least in a *Salmonella* infection model. It would be worthwhile to consider other technical modifications of wheat bran since they may result in even more efficient 'dinner tables'.

EXTENDING THE WHEAT BRAN STRATEGY TO OTHER PLANT BASED ALTERNATIVE FEED INGREDIENTS

Due to the insufficient supply, price volatility and competition with the food and biofuel industry, there is a continuous demand for alternative energy and protein sources for poultry feeds (Ravindran 2013, Leinonen & Kyriazakis 2016, Morgan & Choct 2016, Van Immerseel et al. 2017). Many alternative feedstuffs have obvious potential, but are not frequently used because of constraints imposed by nutritional (e.g. anti-nutritional effects), technical (e.g. need for dehulling and/or processing) and socio-economic (e.g. cost of processing) factors (Ravindran 2013, Leinonen & Kyriazakis 2016, Van Immerseel et al. 2017). Large amounts of grain by-products, such as wheat/rice bran and distillers dried grains with solubles (DDGS), and alternative feedstuffs such as cassava, are available for use in poultry diets all over the world. Often, the main problem with these components is their high content of insoluble NSP (e.g. cellulose and arabinoxylans). Many of their anti-nutritive effects can be overcome by the additional supplementation of exogenous enzymes such as carbohydrases and phytases. Casava for example, is an energy yielding crop produced in tropical regions such as Thailand, which potentially could replace maize in poultry diets completely (Morgan & Choct 2016). It has been shown that the efficiency of nutrient utilization of cassava can be improved by applying microbial enzyme supplements (Bhuiyan & Iji 2015). Paradoxically, the biofuel industry could provide an interesting alternative feed substrate for poultry as well. DDGS have become an abundant by-product from ethanol production. They contain large amounts of NSP but their composition is relatively unknown (Spiehs et al. 2002). The potential of applying enzymes to DDGS for the release of metabolisable energy for monogastric animals is enormous. However, it is often challenging to formulate enzyme preparations that are able to degrade the DDGS NSPs within the transit time of chickens (Choct 2006). Another example of an alternative feedstuff is rice bran, a by-product of rice processing. The main obstacles impeding nutrient utilization of rice bran are phytate, indigestible polysaccharides and ferulic acid (Liu et al. 2015). By adding feruloyl esterase combined with several xylan-degrading enzymes to a feed consisting of 95% rice bran, Liu et al. (2015) could increase the nutrient digestibility *in vivo* and dry matter degradation *in vitro*. By identifying the anti-nutritive factors of alternative feed ingredients and develop optimal enzyme combinations, it is

feasible to increase their digestibility. In this work we have demonstrated an approach, using wheat bran, where one can identify the bacteria that are specifically attached to the substrate and directly degrade it. Then, by characterizing their enzymatic potential, an optimal mixture of bacteria and/or enzymes could be formulated to supplement to the diet containing the alternative feedstuff and increase its digestibility. This way, previously ignored raw materials could be valorized in the future for their use in poultry feeds and the competition between the biofuel, feed and food industry could be reduced.

CONCLUSION

To conclude we would like to present an all-encompassing working mechanism that unifies the different observations made in this work. It seems that wheat bran with reduced particle size is specifically colonized by both lactate producing bacteria from the genera *Bifidobacterium* and *Lactobacillus*, and butyrate producing bacteria from the *Lachnospiraceae* family. The close proximity of these two metabolic groups may enhance their interaction and thus cross-feeding of degradation products such as lactate. Members from the Actinobacteria are known to encode several enzymes involved in xylan degradation and this may attract them to the substrate. The more efficient fermentation of WB280 compared to WB in SCFA may be explained by an increased availability of bran constituents due to the increase in specific surface area. In addition, the presence of a specific butyrogenic community on WB280 might explain the increased production of butyrate as well. For butyrate and propionate it is known that they can negatively affect the expression of *Salmonella* virulence genes. We noticed both a downregulation of the *hilA* gene as well as an actual lowered invasive potential of *Salmonella* bacteria that were pretreated with fermentation products derived from WB280. These observations can explain the increased resilience of the broilers that we observed in the *Salmonella* infection trials. Here we propose the use of WB280 as feed additive, to increase the overall gastrointestinal health of broilers since the supplementation of only 1% can induce several health related effects.

REFERENCES

Amerah, A.M., Ravindran, V., Lentle, R.G. and Thomas, D.G. Feed Particle Size: Implications on the Digestion and Performance of Poultry. (2007) *Worlds Poultry Science Journal* **63**, 3: 439-455.

Atarashi, K., Tanoue, T., Oshima, K., Suda, W., Nagano, Y., Nishikawa, H., Fukuda, S., Saito, T., Narushima, S., Hase, K., Kim, S., Fritz, J.V., Wilmes, P., Ueha, S., Matsushima, K., Ohno, H., Olle, B., Sakaguchi, S., Taniguchi, T., Morita, H., Hattori, M. and Honda, K. Treg Induction by a Rationally Selected Mixture of Clostridia Strains from the Human Microbiota. (2013) *Nature* **500**, 7461: 232-6.

Atarashi, K., Tanoue, T., Shima, T., Imaoka, A., Kuwahara, T., Momose, Y., Cheng, G., Yamasaki, S., Saito, T., Ohba, Y., Taniguchi, T., Takeda, K., Hori, S., Ivanov, II, Umesaki, Y., Itoh, K. and Honda, K. Induction of Colonic Regulatory T Cells by Indigenous Clostridium Species. (2011) *Science* **331**, 6015: 337-41.

Backhed, F., Ley, R.E., Sonnenburg, J.L., Peterson, D.A. and Gordon, J.I. Host-Bacterial Mutualism in the Human Intestine. (2005) *Science* **307**, 5717: 1915-20.

Behnsen, J., Perez-Lopez, A., Nuccio, S.-P. and Raffatellu, M. Exploiting Host Immunity: The Salmonella Paradigm. (2015) *Trends in immunology* **36**, 2: 112-120.

Bhuiyan, M.M. and Iji, P.A. Energy Value of Cassava Products in Broiler Chicken Diets with or without Enzyme Supplementation. (2015) *Asian-Australasian Journal of Animal Sciences* **28**, 9: 1317-1326.

Bohnhoff, M., Miller, C.P. and Martin, W.R. Resistance of the Mouse's Intestinal Tract to Experimental Salmonella Infection. I. Factors Which Interfere with the Initiation of Infection by Oral Inoculation. (1964) *Journal of Experimental Medicine* **120**: 805-16.

Borst, L.B., Suyemoto, M.M., Sarsour, A.H., Harris, M.C., Martin, M.P., Strickland, J.D., Oviedo, E.O. and Barnes, H.J. Pathogenesis of Enterococcal Spondylitis Caused by *Enterococcus Cecorum* in Broiler Chickens. (2016) *Veterinary Pathology* **54**, 1: 61-73.

Buffie, C.G. and Pamer, E.G. Microbiota-Mediated Colonization Resistance against Intestinal Pathogens. (2013) *Nature Reviews Immunology* **13**, 11: 790-801.

Cherrington, C.A., Hinton, M., Pearson, G.R. and Chopra, I. Short-Chain Organic Acids at Ph 5.0 Kill Escherichia Coli and Salmonella Spp. Without Causing Membrane Perturbation. (1991) *Journal of Applied Bacteriology* **70**, 2: 161-5.

Choct, M. Enzymes for the Feed Industry: Past, Present and Future. (2006) *Worlds Poultry Science Journal* **62**, 1: 5-15.

Clench, M.H. The Avian Cecum: Update and Motility Review. (1999) *Journal of Experimental Zoology* **283**, 4-5: 441-447.

Cowieson, A.J. Strategic Selection of Exogenous Enzymes for Corn/Soy-Based Poultry Diets. (2010) *Journal of Poultry Science* **47**, 1: 1-7.

De Paepe, K., Kerckhof, F.M., Verspreet, J., Courtin, C.M. and Van de Wiele, T. Inter-Individual Differences Determine the Outcome of Wheat Bran Colonization by the Human Gut Microbiome. (2017) *Environmental Microbiology*.

De Preter, V., Hamer, H.M., Windey, K. and Verbeke, K. The Impact of Pre- and/or Probiotics on Human Colonic Metabolism: Does It Affect Human Health? (2011) *Molecular Nutrition & Food Research* **55**, 1: 46-57.

de Verdal, H., Mignon-Grasteau, S., Jeulin, C., Le Bihan-Duval, E., Leconte, M., Mallet, S., Martin, C. and Narcy, A. Digestive Tract Measurements and Histological Adaptation in Broiler Lines Divergently Selected for Digestive Efficiency. (2010) *Poultry Science* **89**, 9: 1955-61.

Dhingra, D., Michael, M., Rajput, H. and Patil, R.T. Dietary Fibre in Foods: A Review. (2012) *Journal of Food Science and Technology* **49**, 3: 255-266.

Dodd, D., Mackie, R.I. and Cann, I.K.O. Xylan Degradation, a Metabolic Property Shared by Rumen and Human Colonic Bacteroidetes. (2011) *Molecular Microbiology* **79**, 2: 292-304.

Eeckhaut, V., Wang, J., Van Parys, A., Haesebrouck, F., Joossens, M., Falony, G., Raes, J., Ducatelle, R. and Van Immerseel, F. The Probiotic *Butyricicoccus Pullicaecorum* Reduces Feed Conversion and Protects from Potentially Harmful Intestinal Microorganisms and Necrotic Enteritis in Broilers. (2016) *Frontiers in Microbiology* **7**.

EFSA. The 2013 Joint Ecdc/Efsa Report on Trends and Sources of Zoonoses, Zoonotic Agents and Food-Borne Outbreaks Published. (2015) *EuroSurveillance* **20**, 4.

Evans, S.J. and Sayers, A.R. A Longitudinal Study of *Campylobacter* Infection of Broiler Flocks in Great Britain. (2000) *Preventive Veterinary Medicine* **46**, 3: 209-223.

Ezer, A., Matalon, E., Jindou, S., Borovok, I., Atamna, N., Yu, Z., Morrison, M., Bayer, E.A. and Lamed, R. Cell Surface Enzyme Attachment Is Mediated by Family 37 Carbohydrate-Binding Modules, Unique to *Ruminococcus Albus*. (2008) *Journal of Bacteriology* **190**, 24: 8220-8222.

Flint, H.J., Bayer, E.A., Rincon, M.T., Lamed, R. and White, B.A. Polysaccharide Utilization by Gut Bacteria: Potential for New Insights from Genomic Analysis. (2008) *Nature Reviews Microbiology* **6**, 2: 121-131.

Furusawa, Y., Obata, Y., Fukuda, S., Endo, T.A., Nakato, G., Takahashi, D., Nakanishi, Y., Uetake, C., Kato, K., Kato, T., Takahashi, M., Fukuda, N.N., Murakami, S., Miyauchi, E., Hino, S., Atarashi, K., Onawa, S., Fujimura, Y., Lockett, T., Clarke, J.M., Topping, D.L., Tomita, M., Hori, S., Ohara, O., Morita, T., Koseki, H., Kikuchi, J., Honda, K., Hase, K. and Ohno, H. Commensal Microbe-Derived Butyrate Induces the Differentiation of Colonic Regulatory T Cells. (2013) *Nature* **504**, 7480: 446-50.

Gallaher, D.D. and Khil, J. The Effect of Synbiotics on Colon Carcinogenesis in Rats. (1999) *Journal of Nutrition* **129**, 7 Suppl: 1483S-7S.

Gantois, I., Ducatelle, R., Pasmans, F., Haesebrouck, F., Hautefort, I., Thompson, A., Hinton, J.C. and Van Immerseel, F. Butyrate Specifically Down-Regulates *Salmonella* Pathogenicity Island 1 Gene Expression. (2006) *Applied and Environmental Microbiology* **72**, 1: 946-9.

Gibson, G.R., Probert, H.M., Loo, J.V., Rastall, R.A. and Roberfroid, M.B. Dietary Modulation of the Human Colonic Microbiota: Updating the Concept of Prebiotics. (2004a) *Nutrition Research Reviews* **17**, 2: 259-75.

Gibson, G.R., Probert, H.M., Van Loo, J., Rastall, R.A. and Roberfroid, M.B. Dietary Modulation of the Human Colonic Microbiota: Updating the Concept of Prebiotics. (2004b) *Nutrition Research Reviews* **17**, 2: 259-275.

Hermans, D., Pasmans, F., Messens, W., Martel, A., Van Immerseel, F., Rasschaert, G., Heyndrickx, M., Van Deun, K. and Haesebrouck, F. Poultry as a Host for the Zoonotic Pathogen *Campylobacter Jejuni*. (2012) *Vector-Borne and Zoonotic Diseases* **12**, 2: 89-98.

Hermans, D., Van Deun, K., Messens, W., Martel, A., Van Immerseel, F., Haesebrouck, F., Rasschaert, G., Heyndrickx, M. and Pasmans, F. *Campylobacter* Control in Poultry by Current Intervention Measures Ineffective: Urgent Need for Intensified Fundamental Research. (2011) *Veterinary Microbiology* **152**, 3-4: 219-28.

Hetland, H., Choct, M. and Svihus, B. Role of Insoluble Non-Starch Polysaccharides in Poultry Nutrition. (2004) *Worlds Poultry Science Journal* **60**, 4: 415-422.

Hetland, H., Svihus, B. and Krogdahl, A. Effects of Oat Hulls and Wood Shavings on Digestion in Broilers and Layers Fed Diets Based on Whole or Ground Wheat. (2003) *British Poultry Science* **44**, 2: 275-282.

Hill, C., Guarner, F., Reid, G., Gibson, G.R., Merenstein, D.J., Pot, B., Morelli, L., Canani, R.B., Flint, H.J., Salminen, S., Calder, P.C. and Sanders, M.E. The International Scientific Association for Probiotics and Prebiotics Consensus Statement on the Scope and Appropriate Use of

the Term Probiotic. (2014) *Nature Reviews Gastroenterology & Hepatology* **11**, 8: 506-514.

Hrncırova, M., Pospíšil, J. and Špiláček, M. Size Analysis of Solid Particles Using Laser Diffraction and Sieve Analysis. (2013) *Engineering Mechanics* **20**, 3/4.

Hung, C.C., Garner, C.D., Slauch, J.M., Dwyer, Z.W., Lawhon, S.D., Frye, J.G., McClelland, M., Ahmer, B.M. and Altier, C. The Intestinal Fatty Acid Propionate Inhibits Salmonella Invasion through the Post-Translational Control of Hild. (2013) *Molecular Microbiology* **87**, 5: 1045-60.

Inan, M.S., Rasoulpour, R.J., Yin, L., Hubbard, A.K., Rosenberg, D.W. and Giardina, C. The Luminal Short-Chain Fatty Acid Butyrate Modulates Nf-Kappa B Activity in a Human Colonic Epithelial Cell Line. (2000) *Gastroenterology* **118**, 4: 724-734.

Jacobs, P.J., Bogaerts, S., Hemdane, S., Delcour, J.A. and Courtin, C.M. Impact of Wheat Bran Hydration Properties as Affected by Toasting and Degree of Milling on Optimal Dough Development in Bread Making. (2016a) *Journal of Agricultural and Food Chemistry* **64**, 18: 3636-3644.

Jacobs, P.J., Hemdane, S., Delcour, J.A. and Courtin, C.M. Dry Heat Treatment Affects Wheat Bran Surface Properties and Hydration Kinetics. (2016b) *Food Chemistry* **203**: 513-520.

Jacobs, P.J., Hemdane, S., Dornez, E., Delcour, J. and Courtin, C. Study of Hydration Properties of Wheat Bran as a Function of Particle Size. (2015a) *Food Chemistry* **179**: 296-304.

Jacobs, P.J., Hemdane, S., Dornez, E., Delcour, J.A. and Courtin, C.M. Study of Hydration Properties of Wheat Bran as a Function of Particle Size. (2015b) *Food Chemistry* **179**: 296-304.

Jacobsreitsma, W.F., Vandegiessen, A.W., Bolder, N.M. and Mulder, R.W.A. Epidemiology of *Campylobacter* Spp at 2 Dutch Broiler Farms. (1995) *Epidemiology and Infection* **114**, 3: 413-421.

Jimenez-Moreno, E., Gonzalez-Alvarado, J.M., Gonzalez-Sanchez, D., Lazaro, R. and Mateos, G.G. Effects of Type and Particle Size of Dietary Fiber on Growth Performance and Digestive Traits of Broilers from 1 to 21 Days of Age. (2010) *Poultry Science* **89**, 10: 2197-212.

Jozefiak, T., Larsson, B., Wichstrom, L., Wallander, J. and Mattejat, F. Quality of Life as Reported by Children and Parents: A Comparison between Students and Child Psychiatric Outpatients. (2010) *Health and Quality of Life Outcomes* **8**.

Kalivoda, J.R., Jones, C.K. and Stark, C.R. Impact of Varying Analytical Methodologies on Grain Particle Size Determination. (2017) *Journal of Animal Science* **95**, 1: 113-119.

Kang, J.H. and Lee, M.S. Characterization of a Bacteriocin Produced by Enterococcus Faecium Gm-1 Isolated from an Infant. (2005) *Journal of Applied Microbiology* **98**, 5: 1169-1176.

Kankainen, M., Paulin, L., Tynkkynen, S., von Ossowski, I., Reunanen, J., Partanen, P., Satokari, R., Vesterlund, S., Hendrickx, A.P., Lebeer, S., De Keersmaecker, S.C., Vanderleyden, J., Hamalainen, T., Laukkanen, S., Salovuori, N., Ritari, J., Alatalo, E., Korpela, R., Mattila-Sandholm, T., Lassig, A., Hatakka, K., Kinnunen, K.T., Karjalainen, H., Saxelin, M., Laakso, K., Surakka, A., Palva, A., Salusjarvi, T., Auvinen, P. and de Vos, W.M. Comparative Genomic Analysis of Lactobacillus Rhamnosus Gg Reveals Pili Containing a Human-Mucus Binding Protein. (2009) *Proceedings of the National Academy of Sciences U S A* **106**, 40: 17193-8.

Kim, J.Y., Young, J.A., Gunther, N.W. and Lee, J.L. Inhibition of Salmonella by Bacteriocin-Producing Lactic Acid Bacteria Derived from Us Kimchi and Broiler Chicken. (2015) *Journal of Food Safety* **35**, 1: 1-12.

Kim, M., Qie, Y., Park, J. and Kim, C.H. Gut Microbial Metabolites Fuel Host Antibody Responses. (2016) *Cell Host Microbe* **20**, 2: 202-14.

Knudsen, K.E.B. Fiber and Nonstarch Polysaccharide Content and Variation in Common Crops Used in Broiler Diets. (2014) *Poultry Science* **93**, 9: 2380-2393.

Lai, H.C. and Duke, G.E. Colonic Motility in Domestic Turkeys. (1978) *American Journal of Digestive Diseases* **23**, 8: 673-681.

Lei, F., Yin, Y.S., Wang, Y.Z., Deng, B., Yu, H.D., Li, L.J., Xiang, C., Wang, S.Y., Zhu, B.L. and Wang, X. Higher-Level Production of Volatile Fatty Acids *in Vitro* by Chicken Gut Microbiotas Than by Human Gut Microbiotas as Determined by Functional Analyses. (2012) *Applied and Environmental Microbiology* **78**, 16: 5763-5772.

Leinonen, I. and Kyriazakis, I. How Can We Improve the Environmental Sustainability of Poultry Production? (2016) *Proceedings of the Nutrition Society* **75**, 3: 265-273.

Ley, R.E., Peterson, D.A. and Gordon, J.I. Ecological and Evolutionary Forces Shaping Microbial Diversity in the Human Intestine. (2006) *Cell* **124**, 4: 837-48.

Liu, Q., Zhou, D.Y., Chen, L., Dong, R.Q. and Zhuang, S. Effects of Feruloyl Esterase, Non-Starch Polysaccharide Degrading Enzymes, Phytase, and Their Combinations on in Vitro Degradation of Rice Bran and Nutrient Digestibility of Rice Bran Based Diets in Adult Cockerels. (2015) *Livestock Science* **178**: 255-262.

Lozupone, C.A., Stombaugh, J.I., Gordon, J.I., Jansson, J.K. and Knight, R. Diversity, Stability and Resilience of the Human Gut Microbiota. (2012) *Nature* **489**, 7415: 220-230.

Luethy, P.M., Huynh, S., Ribardo, D.A., Winter, S.E., Parker, C.T. and Hendrixson, D.R. Microbiota-Derived Short-Chain Fatty Acids Modulate Expression of Campylobacter Jejuni Determinants Required for Commensalism and Virulence. (2017) *Mbio* **8**, 3.

Macfarlane, M.J.H.G.T.M.S. Bacterial Growth and Metabolism on Surfaces in the Large Intestine. (2000) *Microbial Ecology in Health and Disease* **12**, 2: 64-72.

Makras, L. and De Vuyst, L. The *in Vitro* Inhibition of Gram-Negative Pathogenic Bacteria by Bifidobacteria Is Caused by the Production of Organic Acids. (2006) *International Dairy Journal* **16**, 9: 1049-1057.

Matter, K., Aijaz, S., Tsapara, A. and Balda, M.S. Mammalian Tight Junctions in the Regulation of Epithelial Differentiation and Proliferation. (2005) *Current Opinion in Cell Biology* **17**, 5: 453-458.

McNulty, N.P., Wu, M., Erickson, A.R., Pan, C.L., Erickson, B.K., Martens, E.C., Pudlo, N.A., Muegge, B.D., Henrissat, B., Hettich, R.L. and Gordon, J.I. Effects of Diet on Resource Utilization by a Model Human Gut Microbiota Containing *Bacteroides Cellulosilyticus* Wh2, a Symbiont with an Extensive Glycobiome. (2013) *Plos Biology* **11**, 8.

Meyer-Hoffert, U., Hornef, M.W., Henriques-Normark, B., Axelsson, L.G., Midtvedt, T., Putsep, K. and Andersson, M. Secreted Enteric Antimicrobial Activity Localises to the Mucus Surface Layer. (2008) *Gut* **57**, 6: 764-71.

Middelbos, I.S., Boler, B.M.V., Qu, A., White, B.A., Swanson, K.S. and Fahey, G.C. Phylogenetic Characterization of Fecal Microbial Communities of Dogs Fed Diets with or without Supplemental Dietary Fiber Using 454 Pyrosequencing. (2010) *Plos One* **5**, 3.

Mignon-Grasteau, S., Narcy, A., Rideau, N., Chantry-Darmon, C., Boscher, M.Y., Sellier, N., Chabault, M., Konsak-Ilievski, B., Le Bihan-Duval, E. and Gabriel, I. Impact of Selection for Digestive Efficiency on Microbiota Composition in the Chicken. (2015) *PLoS One* **10**, 8: e0135488.

Morais, S., Ben David, Y., Bensoussan, L., Duncan, S.H., Koropatkin, N.M., Martens, E.C., Flint, H.J. and Bayer, E.A. Enzymatic Profiling of Cellulosomal Enzymes from the Human Gut Bacterium, *Ruminococcus Champanellensis*, Reveals a Fine-Tuned System for Cohesin-Dockerin Recognition. (2016) *Environmental Microbiology* **18**, 2: 542-56.

Morgan, N.K. and Choct, M. Cassava: Nutrient Composition and Nutritive Value in Poultry Diets. (2016) *Animal Nutrition* **2**, 4: 253-261.

Ng, K.M., Ferreyra, J.A., Higginbottom, S.K., Lynch, J.B., Kashyap, P.C., Gopinath, S., Naidu, N., Choudhury, B., Weimer, B.C., Monack, D.M. and Sonnenburg, J.L. Microbiota-Liberated Host Sugars Facilitate Post-Antibiotic Expansion of Enteric Pathogens. (2013) *Nature* **502**, 7469: 96-+.

Perumal, V., Repally, A., Dasari, A. and Venkatesan, A. Partial Purification and Characterization of Bacteriocin Produced by Enterococcus Faecalis Du10 and Its Probiotic Attributes. (2016) *Preparative Biochemistry and Biotechnology* **46**, 7: 686-94.

Ravindran, V. "Poultry Feed Availability and Nutrition in Developing Countries." In *FAO*, 67-69. Rome, Italy, **2013**.

Rivera-Chavez, F., Zhang, L.F., Faber, F., Lopez, C.A., Byndloss, M.X., Olsan, E.E., Xu, G.G., Velazquez, E.M., Lebrilla, C.B., Winter, S.E. and Baumler, A.J. Depletion of Butyrate-Producing Clostridia from the Gut Microbiota Drives an Aerobic Luminal Expansion of *Salmonella*. (2016) *Cell Host & Microbe* **19**, 4: 443-454.

Roberfroid, M., Gibson, G.R., Hoyles, L., McCartney, A.L., Rastall, R., Rowland, I., Wolvers, D., Watzl, B., Szajewska, H., Stahl, B., Guarner, F., Respondek, F., Whelan, K., Coxam, V., Davicco, M.J., Leotoing, L., Wittrant, Y., Delzenne, N.M., Cani, P.D., Neyrinck, A.M. and Meheust, A. Prebiotic Effects: Metabolic and Health Benefits. (2010) *British Journal of Nutrition* **104**: S1-S63.

Rogowski, A., Briggs, J.A., Mortimer, J.C., Tryfona, T., Terrapon, N., Lowe, E.C., Basle, A., Morland, C., Day, A.M., Zheng, H.J., Rogers, T.E., Thompson, P., Hawkins, A.R., Yadav, M.P., Henrissat, B., Martens, E.C., Dupree, P., Gilbert, H.J. and Bolam, D.N. Glycan Complexity Dictates Microbial Resource Allocation in the Large Intestine (Vol 6, 7481, 2015). (2016) *Nature Communications* **7**.

Rolfe, R.D. Role of Volatile Fatty Acids in Colonization Resistance to Clostridium Difficile. (1984) *Infection and Immunity* **45**, 1: 185-91.

Rougiere, N., Malbert, C.H., Rideau, N., Cognie, J. and Carre, B. Comparison of Gizzard Activity between Chickens from Genetic D+ and D- Lines Selected for Divergent Digestion Efficiency. (2012) *Poultry Science* **91**, 2: 460-7.

Schrezenmeir, J. and de Vrese, M. Probiotics, Prebiotics, and Synbiotics--Approaching a Definition. (2001) *The American Journal of Clinical Nutrition* **73**, 2 Suppl: 361S-364S.

Shin, R., Suzuki, M. and Morishita, Y. Influence of Intestinal Anaerobes and Organic Acids on the Growth of Enterohaemorrhagic Escherichia Coli O157:H7. (2002) *Journal of Medical Microbiology* **51**, 3: 201-6.

Slavin, J. Fiber and Prebiotics: Mechanisms and Health Benefits. (2013) *Nutrients* **5**, 4: 1417-1435.

Sonnenburg, J.L., Chen, C.T. and Gordon, J.I. Genomic and Metabolic Studies of the Impact of Probiotics on a Model Gut Symbiont and Host. (2006) *PLoS Biology* **4**, 12: e413.

Spiehs, M.J., Whitney, M.H. and Shurson, G.C. Nutrient Database for Distiller's Dried Grains with Solubles Produced from New Ethanol Plants in Minnesota and South Dakota. (2002) *Journal of Animal Science* **80**, 10: 2639-2645.

Stanley, D., Hughes, R.J. and Moore, R.J. Microbiota of the Chicken Gastrointestinal Tract: Influence on Health, Productivity and Disease. (2014) *Applied Microbiology and Biotechnology* **98**, 10: 4301-10.

Stecher, B., Robbiani, R., Walker, A.W., Westendorf, A.M., Barthel, M., Kremer, M., Chaffron, S., Macpherson, A.J., Buer, J., Parkhill, J., Dougan, G., von Mering, C. and Hardt, W.D. Salmonella Enterica Serovar Typhimurium Exploits Inflammation to Compete with the Intestinal Microbiota. (2007) *PLoS Biology* **5**, 10: 2177-89.

Stewart, M.L. and Slavin, J.L. Particle Size and Fraction of Wheat Bran Influence Short-Chain Fatty Acid Production *in Vitro*. (2009) *British Journal of Nutrition* **102**, 10: 1404-7.

Svetoch, E.A., Eruslanov, B.V., Levchuk, V.P., Perelygin, V.V., Mitsevich, E.V., Mitsevich, I.P., Stepanshin, J., Dyatlov, I., Seal, B.S. and Stern, N.J. Isolation of Lactobacillus Salivarius 1077 (Nrrl B-50053) and Characterization of Its Bacteriocin, Including the Antimicrobial Activity Spectrum. (2011) *Applied and Environmental Microbiology* **77**, 8: 2749-2754.

Svihus, B. The Gizzard: Function, Influence of Diet Structure and Effects on Nutrient Availability. (2011) *Worlds Poultry Science Journal* **67**, 2: 207-223.

Svihus, B. Function of the Digestive System. (2014) *Journal of Applied Poultry Research* **23**, 2: 306-314.

Svihus, B., Juvik, E., Hetland, H. and Krogdahl, A. Causes for Improvement in Nutritive Value of Broiler Chicken Diets with Whole Wheat Instead of Ground Wheat. (2004a) *British Poultry Science* **45**, 1: 55-60.

Svihus, B., Juvik, E., Hetland, H. and Krogdahl, A. Causes for Improvement in Nutritive Value of Broiler Chicken Diets with Whole Wheat Instead of Ground Wheat. (2004b) *Br Poult Sci* **45**, 1: 55-60.

Timbermont, L., Lanckriet, A., Dewulf, J., Nollet, N., Schwarzer, K., Haesebrouck, F., Ducatelle, R. and Van Immerseel, F. Control of *Clostridium Perfringens*-Induced Necrotic Enteritis in Broilers by Target-Released Butyric Acid, Fatty Acids and Essential Oils. (2010) *Avian Pathology* **39**, 2: 117-121.

Torok, V.A., Dyson, C., McKay, A. and Ophel-Keller, K. Quantitative Molecular Assays for Evaluating Changes in Broiler Gut Microbiota Linked with Diet and Performance. (2013) *Animal Production Science* **53**, 12: 1260-1268.

Tran, T.S., Narcy, A., Carre, B., Gabriel, I., Rideau, N., Gilbert, H., Demeure, O., Bed'Hom, B., Chantry-Darmon, C., Boscher, M.Y., Bastianelli, D., Sellier, N., Chabault, M., Calenge, F., Le Bihan-Duval, E., Beaumont, C. and Mignon-Grasteau, S. Detection of Qtl Controlling Digestive Efficiency and Anatomy of the Digestive Tract in Chicken Fed a Wheat-Based Diet. (2014) *Genetics Selection Evolution* **46**: 25.

Van Deun, K., Haesebrouck, F., Van Immerseel, F., Ducatelle, R. and Pasmans, F. Short-Chain Fatty Acids and L-Lactate as Feed Additives to Control *Campylobacter Jejuni* Infections in Broilers. (2008a) *Avian Pathology* **37**, 4: 379-383.

Van Deun, K., Pasmans, F., Van Immerseel, F., Ducatelle, R. and Haesebrouck, F. Butyrate Protects Caco-2 Cells from *Campylobacter Jejuni* Invasion and Translocation. (2008b) *British Journal of Nutrition* **100**, 3: 480-484.

Van Houdt, R. and Michiels, C.W. Role of Bacterial Cell Surface Structures in *Escherichia Coli* Biofilm Formation. (2005) *Research in Microbiology* **156**, 5-6: 626-33.

Van Immerseel, F., Eeckhaut, V., Moore, R.J., Choct, M. and Ducatelle, R. Beneficial Microbial Signals from Alternative Feed Ingredients: A Way to Improve Sustainability of Broiler Production? (2017) *Microbial Biotechnology*.

Van Immerseel, F., Russell, J.B., Flythe, M.D., Gantois, I., Timbermont, L., Pasmans, F., Haesebrouck, F. and Ducatelle, R. The Use of Organic Acids to Combat Salmonella in Poultry: A Mechanistic Explanation of the Efficacy. (2006a) *Avian Pathology* **35**, 3: 182-188.

Van Immerseel, F., Russell, J.B., Flythe, M.D., Gantois, I., Timbermont, L., Pasmans, F., Haesebrouck, F. and Ducatelle, R. The Use of Organic Acids to Combat *Salmonella* in Poultry: A Mechanistic Explanation of the Efficacy. (2006b) *Avian Pathology* **35**, 3: 182-8.

Vermeulen, K., Verspreet, J., Courtin, C.M., Haesebrouck, F., Ducatelle, R. and Van Immerseel, F. Reduced Particle Size Wheat Bran Is Butyrogenic and Lowers *Salmonella* Colonization When Added to Poultry Feed. (2017) *Veterinary Microbiology* **198**: 64-71.

Verspreet, J., Damen, B., Broekaert, W.F., Verbeke, K., Delcour, J.A. and Courtin, C.M. A Critical Look at Prebiotics within the Dietary Fiber Concept. (2016) *Annual Review of Food Science and Technology* **7**: 167-90.

Walker, A.W., Duncan, S.H., Harmsen, H.J., Holtrop, G., Welling, G.W. and Flint, H.J. The Species Composition of the Human Intestinal Microbiota Differs between Particle-Associated and Liquid Phase Communities. (2008) *Environmental Microbiology* **10**, 12: 3275-83.

Wang, H., Wang, P., Wang, X., Wan, Y. and Liu, Y. Butyrate Enhances Intestinal Epithelial Barrier Function Via up-Regulation of Tight Junction Protein Claudin-1 Transcription. (2012) *Digestive Diseases and Sciences* **57**, 12: 3126-3135.

Wideman, R.F., Jr. Bacterial Chondronecrosis with Osteomyelitis and Lameness in Broilers: A Review. (2016) *Poultry Science* **95**, 2: 325-44.

Wilkins, T. and Sequoia, J. Probiotics for Gastrointestinal Conditions: A Summary of the Evidence. (2017) *American Family Physician* **96**, 3: 170-178.

Windey, K., De Preter, V. and Verbeke, K. Relevance of Protein Fermentation to Gut Health. (2012) *Molecular Nutrition & Food Research* **56**, 1: 184-96.

Zhao, L., Wang, G., Siegel, P., He, C., Wang, H., Zhao, W., Zhai, Z., Tian, F., Zhao, J., Zhang, H., Sun, Z., Chen, W., Zhang, Y. and Meng, H. Quantitative Genetic Background of the Host Influences Gut Microbiomes in Chickens. (2013) *Scientific Reports* **3**: 1163.

PART 5

APPENDICES

Summary

SUMMARY

In industrial poultry production, eggs are separated from the parental flock prior to hatching. This reduces the parental influence on the microbiota composition, resulting in an underdeveloped intestinal microbiota and immune system. In addition, during the rearing period birds are placed in confined and stressful environments. All of these factors make the animals more susceptible to bacterial infections such as *Salmonella*. Chickens are often infected with *Salmonella* at an early age and these infections tend to persist until the age of slaughter, enabling spread into the food chain. The crucial role of the microbiota in the gastrointestinal tract for host well-being and health is well-recognized. There is an increasing awareness of society and the feed/food industry for the possibility and need to promote animal well-being through healthy and functional high quality food and feed. For a while already, the search for appropriate feed additives that can improve the composition and activity of the intestinal microbial community is going on. Wheat bran is a relatively cheap byproduct of the milling of wheat into flour and consists of both fermentable and less fermentable fibres, providing nutrients and an attachment site for bacteria, respectively. These fibres reach the hindgut undigested where they are (partially) fermented by the resident microbiota. A large part of the health promoting effects of dietary fibre can be attributed to their fermentation into short chain fatty acids (SCFA). Wheat bran can be easily technically modified and since technologies employed to alter wheat bran are becoming increasingly available to the industry, one can envisage modifying wheat bran properties to yield an optimal impact on the gut microbiota. Therefore, the general aim of this thesis was to investigate whether technically modified wheat bran could be a valuable tool to induce beneficial shifts in the cecal community composition of broilers and potentially improve their overall health.

In a **first study**, different technically modified wheat bran fractions were *in vitro* fermented with broiler cecal contents. After 24h the bran material was washed to remove all non- and loosely adhered bacteria. The DNA of the strongly adhered community was extracted and subjected to 16S rRNA amplicon sequencing. Data analysis revealed that the particle-associated community and the free-living bacterial

community differ significantly. The community attached to wheat bran was enriched in putative AX degrading taxa The relative abundance of *Enterobacteriaceae*, containing important broiler pathogens such as *Salmonella*, was significantly reduced on non-modified wheat bran (WB) and even more on wheat bran with an average reduced particle size of 280 µm (WB280). In addition, the different bran fractions were colonized by very distinct communities. WB280 was enriched with lactate producing taxa situated within the *Lactobacillaceae* and *Bifidobacteriaceae* families and lactate-consuming, butyrate-producing *Lachnospiraceae*. The close proximity of these two functional groups on wheat bran is hypothesized to promote cross-feeding on lactate and possibly other degradation and fermentation products. When WB280 was administered to broilers, similar shifts could be observed in their cecal content: numbers of *Enterobacteriaceae* declined significantly while the total number of butyrate producing taxa (*Ruminococcaceae* + *Lachnospiraceae*) increased.

In a **second study** we investigated whether the application of wheat bran fractions with different average particle sizes could protect against cecal pathogen colonization, using *Salmonella* as a model challenge organism. Therefore broilers were fed 1 % wheat bran with average particle sizes of 1690 µm (WB), 1200 µm, 520 µm, 280 µm (WB280), 150 µm or 80 µm and challenged with *Salmonella* Enteritidis (SE147). WB280 significantly reduced cecal colonization and fecal shedding shortly after infection. Using batch fermentations, we found that WB280 was more efficiently fermented in butyrate and propionate compared to regular wheat bran. The expression of *hilA*, a regulator of intestinal epithelial cell invasion, was studied by means of a lux reporter system which was introduced in SE147. Incubation of *Salmonella* bacteria with fermentation products derived from WB280 fermented with cecal contents, induced a downregulation of the invasion gene *hilA*. The expression of hilA was studied by means of a *hilA*-lux reporter system which was introduced in SE147. It is generally accepted that butyrate and propionate are capable of affecting *hilA* expression. The downregulation of the *hilA* gene was reflected in an actual lowered invasion potential by *Salmonella* in a colon carcinoma cell line. These observations may explain why WB280 increases the resilience of chickens against *Salmonella* infections *in vivo*, as invasion has been shown to be correlated with

gut colonization. Further investigation is needed to verify whether, besides butyrate and propionate, other unidentified compounds are involved.

In a **third study** we tested whether the results obtained by supplementing WB280 to the feed of *Salmonella* challenged broilers could be mimicked by delivering two members of the community which was specifically attached to WB280. In the first experimental study we identified two Operational taxonomic units (OTUs) that were enriched on WB280: an OTU showing 99% sequence similarity with *Bifidobacterium pseudolongum* and an OTU showing 97% sequence similarity with *Ruminococcus torques*. These strains were administered to broilers, separately and combined, where after the animals were challenged with SE147. Both strains reduced cecal colonization compared to the control chickens. The combined administration showed a synergistic effect resulting in even lower cecal *Salmonella* counts. *In vitro* work using the *hilA*-lux reporter system, showed that in the presence of WB both strains produce metabolites that reduce invasion gene expression by *Salmonella*. In the presence of WB280 the anti-*Salmonella* activity was even more pronounced. Further research is needed to fully understand the mechanism of action and the responsible metabolites should be identified and characterized.

To conclude, it seems that wheat bran with reduced particle size is specifically colonized by both lactate producing bacteria from the genera *Bifidobacterium* and *Lactobacillus*, and lactate consuming, butyrate producing bacteria from the *Lachnospiraceae* family. The close proximity of these two metabolic groups may enhance their interaction and cross-feeding of degradation- and fermentation products. Members from the Actinobacteria are known to encode several enzymes involved in xylan degradation and this may aid in the association with the substrate. The more efficient fermentation of WB280 compared to WB in SCFA may be explained by an increased availability of bran constituents due to the increase in specific surface area. In addition, the presence of a specific butyrogenic community on WB280 might explain the increased production of butyrate as well. For butyrate and propionate it is known that they can negatively affect the expression of *Salmonella* virulence genes. We noticed both a downregulation of the *hilA* promoter as well as an actual lowered invasive potential of *Salmonella* bacteria that were incubated with fermentation products derived from WB280. These observations can explain the increased resilience of the broilers that we observed in the infection

trials. Here we propose the use of WB280 as feed additive to increase the overall gastrointestinal health of broilers since the supplementation of only 1% could induce several health related effects.

SAMENVATTING

SAMENVATTING

In de industriële pluimveehouderij worden eieren geïsoleerd van de moederdieren voordat ze worden uitgebroed. Dit verkleint de invloed van de moederdieren op de microbiota samenstelling, resulterend in een onderontwikkeld darmmicrobioom en immuunsysteem. Daarnaast worden de dieren tijdens de opfok vaak ondergebracht in een besloten, stresserende omgeving. Al deze factoren samen zorgen ervoor dat de dieren vatbaarder zijn voor bacteriële infecties zoals bv. *Salmonella*. Kippen raken vaak al op jonge leeftijd geïnfecteerd met *Salmonella*. Deze infecties houden over het algemeen aan tot slachtleeftijd waardoor de bacteriën potentieel geïntroduceerd worden in de voedselketen. Het feit dat de darmmicrobiota een cruciale rol speelt in de algemene gezondheid van de gastheer is algemeen erkend. Zowel de samenleving als de voederindustrie worden er zich meer en meer van bewust dat gezond en functioneel hoogkwalitatieve voeders noodzakelijk zijn ter bevordering van de algemene gezondheid. De zoektocht naar geschikte voederadditieven die de samenstelling en activiteit van de darmmicrobiota kunnen bevorderen, is al geruime tijd aan de gang. Tarwezemelen zijn een relatief goedkoop bijproduct van de tarwebloemproductie. Ze zijn een hooggeconcentreerde bron van goed en minder goed fermenteerbare vezels die bacteriën kunnen voorzien in respectievelijk nutriënten en aanhechtingsplaatsen. Deze vezels zijn onverteerbaar en bereiken als dusdanig het distale deel van de darm. Daar worden ze (gedeeltelijk) gefermenteerd door de residentiële microbiota. Het overgrote deel van de gezondheidsbevorderende effecten die worden toegeschreven aan deze vezels zijn te wijten aan het feit dat ze gefermenteerd worden tot korte keten vetzuren. Tarwezemelen kunnen technisch gezien op relatief eenvoudige manier gemodificeerd worden en de vereiste technologieën zijn tegenwoordig toegankelijk voor de industrie. Dit betekent dat deze technologieën efficiënter en frequenter toegepast kunnen worden om de intrinsieke eigenschappen van tarwezemelen zodanig te wijzigen dat ze een optimale impact hebben op de darmmicrobiota. Bijgevolg kan de algemene onderzoeksvraag van deze thesis als volgt worden geformuleerd: *Kunnen technisch gemodificeerde tarwezemelen van betekenis zijn bij het sturen en promoten van een*

gezonde cecale darmmicrobiota die daarenboven de algemene gezondheid van vleeskippen kan stimuleren?

In een **eerste studie** werden verschillend gemodificeerde tarwezemelen *in vitro* gefermenteerd met de cecale inhoud van vleeskippen. Na 24u werden de zemelen gewassen om de niet- en zwak aangehechte bacteriën te verwijderen. Het DNA van de sterk aangehechte bacteriën werd geëxtraheerd en opgestuurd voor 16S rRNA amplicon sequencing. Analyse van de resulterende data toonde aan dat de partikel-geassocieerde gemeenschap en de vrijlevende gemeenschap sterk van elkaar verschillen. De bacteriele gemeenschap die specifiek was aangehecht aan tarwezemelen was verrijkt aan arabinoxylaan degraderende taxa. De relatieve abundantie van *Enterobacteriaceae* was significant afgenomen op ongemodificeerde tarwezemelen (WB) en zelfs nog meer uitgesproken op tarwezemelen met een gemiddelde gereduceerde partikelgrootte van 280 µm (WB280). Daarnaast werden deze twee fracties door verschillende bacteriële gemeenschappen gekoloniseerd. Zo was WB280 verrijkt aan melkzuurproducerende bacteriën van de families *Lactobacillaceae* en *Bifidobacteriaceae* en boterzuurproducerende bacteriën van de familie *Lachnospiraceae*. De nabijheid van deze twee functionele groepen op WB280 zou mogelijks onderlinge cross-feeding van melkzuur en andere fermentatie- en degradatieproducten kunnen bevorderen. Er konden gelijkaardige verschuivingen worden waargenomen in het cecum van vleeskippen wanneer WB280 werd gesupplementeerd aan het voeder: *Enterobacteriaceae* aantallen namen significant af en het totaal aantal boterzuurproduceerders (*Lachnospiraceae* + *Ruminococcacaea*) nam toe ten opzichte van een controlegroep kippen die ongesupplementeerd voeder kreeg. De inname van vezelrijke producten wordt vaak gelinkt aan een toegenomen aantal Firmicutes en afname van Proteobacteria. Hier toonden we aan dat we deze shift kunnen bewerkstelligen met slechts 1% WB280.

In een **tweede studie** onderzochten we of de toediening van tarwezemelen met verschillende gemiddelde partikelgrootte bescherming kan bieden tegen vleeskip pathogenen. Daarvoor werd gebruik gemaakt van een *Salmonella* infectiemodel. Het voeder van kippen werd gesupplementeerd met 1% tarwezemel met respectievelijke gemiddelde grootte van 1690 (WB), 1200, 520, 280 (WB280), 150 of 80 µm. Vervolgens

werden de dieren geïnfecteerd met *Salmonella* Enteritidis SE147. Supplementatie van WB280 kon kort na infectie de cecale kolonisatie en fecale uitscheiding significant reduceren vergeleken met de controlegroep. Vervolgens werden de zemelenfracties *in vitro* gefermenteerd en werd de productie van korte keten vetzuren opgevolgd. WB280 werd efficiënter omgezet in boterzuur en propionzuur vergeleken met WB. Met behulp van een lux reportersysteem werd de expressie van het *Salmonella* invasiegen *hilA* nagegaan. Wanneer *Salmonella* werd behandeld met fermentatieproducten afgeleid van WB280, kon een reductie in *hilA* expressie worden waargenomen. Van boterzuur en propionzuur is geweten dat ze de expressie van *hilA* kunnen beïnvloeden. De neerregulatie van *hilA* induceerde een effectieve verlaging van het invasiepotentieel van *Salmonella* bacteriën. Deze observaties bieden mogelijk een verklaring voor de geobserveerde verhoogde resiliëntie van vleeskippen tegen *Salmonella in vivo*. Verder onderzoek is echter vereist om na te gaan of, naast boterzuur en propionzuur, nog andere metabolieten van belang zijn.

In een **derde studie** testten we of de resultaten bekomen uit de infectieproeven met WB280 herhaald konden worden door het aanleveren van twee leden van de gemeenschap specifiek die aangehecht was op WB280. In de eerste studie identificeerden we twee *operational taxonomic units* (OTUs) die respectievelijk 99% en 97% sequentiesimilariteit vertoonden met *Bifidobacterium pseudolongum* en *Ruminococcus torques*. Deze kiemen werden apart en gecombineerd toegediend aan vleeskippen waarna de dieren geïnfecteerd werden met *Salmonella* Enteritidis SE147. De enkelvoudige toediening van de twee kiemen resulteerde in verlaagde cecale *Salmonella* aantallen vergeleken met de controle. De gecombineerde toediening resulteerde in een synergistisch effect. *In vitro* werd het effect van de twee kiemen en hun combinatie nagegaan op *hilA* expressie. In de aanwezigheid van WB induceerden beide stammen een reductie in *hilA* expressie. Dit effect was nog meer uitgesproken in de aanwezigheid van WB280. Om het onderliggende mechanisme bloot te leggen is verder onderzoek noodzakelijk. De verantwoordelijke metabolieten moeten gekarakteriseerd en geïdentificeerd worden.

Om te concluderen kunnen we stellen dat tarwezemelen met een gereduceerde partikelgrootte specifiek worden gekoloniseerd door melkzuurproducerende bacteriën

van de genera *Lactobacillus* en *Bifidobacterium* en boterzuurproduceerders van de *Lachnospiraceae* familie. De nabijheid van deze twee functionele groepen kan potentieel interactie en cross-feeding van fermentatie en degradatieproducten bevorderen. Bepaalde leden van de Actinobacteria zijn gekend voor de productie van enzymen betrokken bij xylaan degradatie en dit trekt hun mogelijk aan tot het substraat. Vergeleken met WB wordt WB280 efficiënter gefermenteerd in korte keten verzuren. Dit kan te maken hebben met de verhoogde toegankelijkheid van de zemelcomponenten door de toename in specifiek oppervlak. Daarnaast kan ook de aanwezigheid van de butyrogene gemeenschap op WB280 de verhoogde boterzuurproductie mogelijk (deels) verklaren. Van boterzuur en propionzuur is geweten dat ze een negatieve impact kunnen hebben op de expressie van *Salmonella* virulentiegenen. Er kon een neerregulatie van de *hilA* promotor geobserveerd worden en de daarmee gepaard gaande reductie in invasiepotentieel wanneer *Salmonella* bacteriën werden geïncubeerd met fermentatieproducten afgeleid van WB280. Al deze observaties bieden een mogelijke verklaring voor de verhoogde resiliëntie die waargenomen werd tijdens de *Salmonella* infectieproeven na toediening van WB280. We stellen hier het gebruik voor van WB280 als voederadditief om zowel de gastro-intestinale als algemene gezondheid van vleeskippen te bevorderen aangezien de supplementatie van slechts 1% verscheidene gezondheidgerelateerde effecten kon induceren.

BIBLIOGRAPHY

SCIENTIFIC PUBLICATIONS

Vermeulen K., Verspreet J., Courtin C.M., Haesebrouck F., Baeyen S., Haegeman A., Ducatelle R., Van Immerseel F. Reduced particle size wheat bran is colonized by a butyrogenic community and alters the cecal microbiota composition of broilers (Submitted).

Vermeulen K., Verspreet J., Courtin C.M., Haesebrouck F., Ducatelle R., Van Immerseel F. (2017). Reduced particle size wheat bran is butyrogenic and lowers *Salmonella* colonization, when added to poultry feed. *Vet. Microbiol*. 198:64-71.

Boets E., Gomand S.V., Deroover L., Preston T., **Vermeulen K.**, De Preter V., Hamer H.M., Van den Mooter G., De Vuyst L., Courtin C.M., Annaert P., Delcour J.A., Verbeke K.A. (2017). Systemic availability and metabolism of colonic-derived short-chain fatty acids in healthy subjects - a stable isotope study. J. Physiol 595(2): 541-555.

Onrust L., Ducatelle R., Van Driessche K., De Maesschalck C., **Vermeulen K.**, Haesebrouck F., Eeckhaut V., Van Immerseel F. (2015). Steering endogenous butyrate production in the intestinal tract of broilers as a tool to improve gut health. *Front. Vet Sci*. 2:75.

Boets E., Deroover L., Houben E., **Vermeulen K.**, Gomand S.V., Delcour J.A., Verbeke K.A. (2015). Quantification of *in vivo* colonic short chain fatty acid production from inulin. *Nutrients* 7 (11): 8916-29.

Verherstraeten S., Goossens E., Valgaeren B., pardon B., Timbermont L., **Vermeulen K.**, Schauvliege S., Haesebrouck F., Ducatelle R., Dprez P., Van Immerseel F. (2013). The synergistic necrohemorrhagic action of *Clostridium perfringens* perfringolysin and alpha toxin in the bovine intestine and against bovine endothelial cells. *Vet Res*. 44:45

CONFERENCE CONTRIBUTIONS

Vermeulen K., Verspreet J., Courtin C.M., Haesebrouck F., Baeyen S., Haegeman A., Ducatelle R., Van Immerseel F. Small particle size wheat bran suppresses *Salmonella* colonization in broilers by hosting a butyrogenic microbial network. (2017). 5th IHSIG Symposium on Poultry Gut Health, 11-12 October 2017, Bangkok.

Antonissen G., Van Immerseel F., Michiels A., Eeckhaut V., **Vermeulen K**., Reisinger N., Baeyen S., Haegeman A., Audenaert K., De Saeger S., Maes D., Haesebrouck F., Ducatelle R. and Croubels S. Effect of feed-borne *Fusarium* mycotoxins on the gut microbiome composition in broiler chickens and in pigs. 1st Mycokey Internation Conference. Global Mycotoxin Reduction in the Food and Feed Chain, 11-14 September 2017, Ghent.

Antonissen G., Croubels S., Eeckhaut V., Reisinger N., Baeyen S., Haegeman A., **Vermeulen K.**, Haesebrouck F., Ducatelle R., Van Immerseel F. (2017) Mycotoxins deoxynivalenol and fumonisisn modulate the intestinal microbiota in broiler chickens. WVPA XXth Congress, 4-8 September 2017, Edinburgh.

Vermeulen K., Verspreet J., Courtin C.M., Baeyen S., Haegeman A., Ducatelle R., Van Immerseel F. (2017) Small particle size wheat bran suppresses *Salmonella* colonization in broilers by hosting a butyrogenic microbial network. 21st European Symposium on Poultry Nutrition, 8-11 May 2017, Salou.

Van Immerseel F., **Vermeulen K.**, Onrust L., Eeckhaut V., Ducatelle R. (2017). Nutritional modulation of microbial signals in the distal intestinal tract and how they can affect broiler health. 21st European Symposium on Poultry Nutrition, 8-11 May 2017, Salou.

Vermeulen K., Verspreet J., Courtin C.M., Haesebrouck F., Ducatelle R., Van Immerseel F. (2017). Reduced particle size wheat bran is butyrogenic and lowers *Salmonella* virulence when added to poultry feed. HEALTHGRAIN Forum Spring Meeting 2017, 2-3 May 2017, Leuven.

Vermeulen K., Verspreet J., Courtin C.M., Haesebrouck F., Ducatelle R., Van Immerseel F. (2016). Reduced particle size wheat bran is butyrogenic and lowers *Salmonella* virulence when added to poultry feed. 4th IHSIG Symposium on Poultry Gut Health, 26-27 October 2016, São Paulo.

CURRICULUM VITAE

PERSONALIA

NAME:	Karen Vermeulen
DATE OF BIRTH:	April 7, 1988
PLACE OF BIRTH:	Geel, Belgium
NATIONALITY:	Belgian

EDUCATION

2000-2006	Secondary school, Latin – Sciences St. Dimpnacollege, Geel, Belgium
2006-2009	Bachelor of Science Biology Ghent University, Gent, Belgium
2009-2010	Preparatory program Master of Science Biochemistry & Biotechnology Ghent University, Gent, Belgium
2010-2012	Master of Science in Biochemistry & Biotechnology Thesis: *"Studie naar de pathogenese van enterotoxemie bij kalveren"* **Promotor**: Prof. Dr. Filip. Van Immerseel Department of Pathology, Bacteriology & Avian Diseases Ghent University, Gent, Belgium
2012-2013	Scientific researcher Subject: "*Onderdrukken van sulfaatreducerende bacteriën bij patiënten met ulceratieve colitis m.b.v. prebiotica*" Department of Pathology, Bacteriology & Avian Diseases Ghent University, Gent, Belgium
2014-2017	PhD in Veterinary Sciences Thesis: "*Wheat bran with reduced particle size as carrier for microbial networks that affect Salmonella colonization*" **Promotor**: Prof. Dr. Filip Van Immerseel **Co-promotor**: Prof. Dr. Richard Ducatelle Department of Pathology, Bacteriology & Avian Diseases Ghent University, Gent, Belgium

DANKWOORD

Gedurende de afgelopen vijf jaar heb ik het geluk gehad om met een heleboel sympathieke mensen te mogen samenwerken. Uiteraard kan een uitgebreid dankwoord niet uitblijven.

In eerste instantie zou ik mijn twee fantastische promotoren willen bedanken. **Prof. Van Immerseel**, **Filip**, & **Prof. Ducatelle**, vooreerst wil ik jullie bedanken voor de vele kansen die ik gekregen heb over de jaren heen: eerst was er het project op de sulfaat reducerende bacteriën, daarna hebben jullie mij opnieuw een kans geboden op een doctoraat met het BRANDING project. En nu ook dit op zijn einde loopt, kreeg ik de kans om nog een tijdje langer aan de vakgroep te blijven. Bedankt! Jullie deuren staan (letterlijk & figuurlijk) altijd wagenwijd open. Hoe druk ook, jullie vinden altijd wel tijd, waarvoor dank, want dit is niet vanzelfsprekend. Filip, het is ongelofelijk hoe jij je kan vastbijten in iets als het je interesse wekt. Dag en nacht denk je mee! Bedankt ook dat ik enkele memorabele congressen mocht bijwonen! Een uitspraak die u erg typeert Prof. Ducatelle komt van Isaac Asimov en gaat als volgt: *The most exciting phrase in science is not 'Eureka!' but 'That's funny …'*. Resultaten waar anderen soms moedeloos van zouden worden, wekken bij u net enthousiasme en nieuwsgierigheid op en dat is bewonderenswaardig. Eveneens bedankt voor de peptalk wanneer nodig!

Prof. Haesebrouck, bedankt voor de mogelijkheid om aan het labo te kunnen doctoreren met alle mogelijke middelen. Tevens veel dank voor het kritisch nalezen van de manuscripten.

Prof. Dr. Christophe Courtin & **Prof. Dr. Tom Van de Wiele**, in de eerste plaats bedankt om jullie schouders onder het BRANDING project te zetten. Het was een voorrecht om er deel van uit te maken. Daarnaast wil ik jullie uitdrukkelijk bedanken voor de suggesties, correcties en opmerkingen die dit werk sterk verbeterd hebben. **Prof. Dr. Myriam Hesta**, evenzeer bedankt om mijn thesis kritisch na te lezen. U heeft bepaalde zaken aangehaald die nog onvoldoende waren uitgewerkt of overdacht. **Dr. Gunther Antonissen**, bedankt om mijn thesis zo zorgvuldig na te lezen en dat ik steeds bij je terecht kan voor (kip-)gerelateerde vragen. Moest je nog hulp nodig hebben voor je buitenlandse staalnames, je weet me te vinden ;)
Prof. Dr. Jeroen de Wulf, bedankt dat u de taak van voorzitter op zich heeft willen nemen.

Dit werk werd gefinancierd door het "Vlaams Agentschap Innoveren en Ondernemen" (Vlaio) en werd uitgevoerd in het kader van het SBO BRANDING, waaraan verscheidene onderzoeksgroepen samenwerkten. Ik zou mijn BRANDING collega's dan ook uitdrukkelijk willen bedanken voor de fijne samenwerking. **Dr. Audrey Neyrinck**, **Prof. Dr. Benedikt Sas**, **Dr. Isabelle Sioen**, **Prof. Dr. Jan Delcour**, **Prof. Dr. Kristin Verbeke**, **Dr. Kristof Brijs**, **Dr. Kurt Gebruers**, **Prof. Dr. Luc De Vuyst**, **Prof. Dr. Nathalie Delzenne**, **Prof. Dr. Stefan Weckx**, **Joran**, **Chiara**, **Sami**, **Pieter**, **Elien**, **Kim**, **Francesco**, **David** & **Lise**: We've spent several hours together in meetings and this has led to the fact that we got to know each other better as the project proceeded. I can still remember the first meeting and it is hard to believe that 4 years have passed already. It was nice for each of us to do our own thing on the project and still pursue more or less the same goal. I've learned a lot from you and I admire your skills and expertise. It was a privilege to work with you. I wish you all the best for the future. **Joran**, misschien eens tot in Ten Aard, of bij de volgende halve marathon in Kasterlee! Bedankt om ons te voorzien van kilo's zemelen. Veel succes met je nieuwe job! **Chiara**, bedankt dat je af en toe mijn hulplijn wilde zijn! Succes met je onderzoek! **Sami**, bedankt voor de übercoole SYNbran, en veel succes op je nieuwe werkplek! **Pieter**, bedankt voor de nuttige opmerkingen en suggesties tijdens de BRANDING vergaderingen. **Elien**,

al ben je al even niet meer betrokken bij het BRANDING project, bedankt voor die eerste batch zemelen! Het was leuk om met je samen te werken. **Kim**, het is niet te geloven hoeveel werk jij verzet, dag en nacht lijk je aan de SHIME gekluisterd. Bedankt voor de mooie foto en voor de uitleg i.v.m. de microbioom analyses en heel veel succes met het afronden van je doctoraat! **David**, bedankt voor je hulp met de lactaat bepaling, hoewel er niets is uitgekomen was het tof om je een dagje te volgen in je natuurlijke habitat ;) Succes nog met je complexe fermentatie experimenten. **Francesco**, you are *molto simpatico*, a nice guy to have around! I wish you all the best and lots of success finishing your PhD! And last but not least, **Lise**, jij hebt het voorrecht om de zemelen los te laten op mensen. Super interessant maar het lijkt geen sinecure te zijn: uitgebreide zoektochten naar proefpersonen, lange dagen van hard labeur... Ik hoop oprecht dat je inspanningen beloond worden! Veel succes nog met alles wat je doet!

Als de kippen in de stal weer eens niet wilden meewerken, dan waren er wel nog altijd die kiekens van een collega's om de dag weer goed te maken.

Venessa, jij was daar heel in het begin om mij wegwijs te maken in de wondere wereld van de anaeroben. Tot op vandaag kan ik beroep doen op jouw kennen en kunnen. Bedankt!! Ik heb me rot geamuseerd in Saõ Paulo met jou. Dat etentje bij DOM is iets wat me altijd zal bijblijven. Dat je nog veel lekker mag gaan eten, zonnige reisjes mag maken, kruid mag trekken in je tuin, veel korting kan scoren op vente-exclusive en de schoonste potjes mag bakken! PS, Als er een wereldkampioenschap gasflessen vervangen zou bestaan voor duo's, dan maakten we uitermate veel kans op de gouden plak. **Lonneke**, mijn favoriete hollander! (plaats wel te delen met ene Maxime Mahu) Tijdens de afgelopen vier jaar heb ik twee - echt verschrikkelijke - proeven moeten doen en ik ben zóóó content dat dat samen met jou was. Samen uit, samen huilen (of zoiets). Het was super duper leuk om met jou op congres te gaan naar Bangkok. Tuk tuks volgestapeld met congreszakjes for the win! Ik hoop dat je volgend jaar de tijd van je leven zal beleven terwijl je de wereld rondreist. Dat we samen nog vaak croqueskes mogen eten en jus d'orange mogen drinken ;) **Evy**/Davy, eigenlijk ben ik via jou in een doctoraat gerold. Ik weet dat ik je destijds verrast heb met mijn thesiskeuze, maar ik heb er nog steeds geen spijt van. Je was er tijdens mijn thesisverdediging en opnieuw die laatste minuten voor mijn interne verdediging. De cirkel is dus rond. Het was steeds gezellig om met jou en Celine naar de naailes te gaan; hoewel het soms verschrikkelijk frustrerend was, mis ik het af en toe. We moeten ook eens dringend kijken voor een vervolg op de leuke kajaknamiddag en bbq. Ik wens jou, Koen, Fiebe en Finn het aller-aller-beste toe. **Celine**, jouw vertrek was het begin van het einde van onze coole bureau. Het lijkt nog niet zo lang geleden maar ondertussen is er al heel wat tijd over heen gegaan. Het feit dat je elke morgen eerst op de bureau was, was nog eens een zekerheid in het leven ;) Bedankt om mij wegwijs te maken in het labo, mij een aantal protocols aan te leren, voederzakken leren dichtnaaien etc... Voeder inmengen op het ILVO is nooit meer zo gezellig geweest als toen met jou! Bedankt ook voor de naaitipjes. Het was plezierig om wekelijks samen naar de les te gaan! Mooie kledingstukken heb ik er niet aan overgehouden, leuke herinneringen des te meer! Ik wens je het allerbeste toe, in alles wat je doet! **Wolf**/Wolverine, ik heb altijd graag bij jou op de bureau gezeten. Dat je af en toe in je neus zat te peuteren, nam ik er graag bij. Bedankt om je *Salmonella* kennis door te geven, het is serieus van pas gekomen. Ik hoor dat je het goed stelt op je nieuwe werk. Ik wens je veel heel succes, in alles wat je doet. Toch moet er me nog iets van het hart: mini-voetbal is geen echte voetbal. Onlosmakelijk verbonden met Wolf: **Iris**. Van alle collega's was jij de zachtaardigste! Bedankt voor alles. Veel succes met alles

wat je doet! **Sofie G**/Geeraerts/Gerrybil, ik vond je in het begin maar een irritante, mij beschuldigen van glaasjesdiefstal... geheel onterecht :p Maar ik heb het je vergeven, en later ben ik je zelfs gaan appreciëren ;) 't Was altijd leuk zeveren met jou. Ik denk nog vaak met weemoed terug naar onze oude bureau en jouw stoeltje aan de gerbilbak, gele stressballen die in het rond vlogen,... Laat het weten als er nog eens een fluitconcert aankomt! En laten we nog eens frieten gaan eten in de Pitstop of een concertje meepikken, al dan niet op de Lokerse feesten! PS, ik begrijp je gezaag over samenvattingen nu wel... het spijt me dat ik niet altijd even begripvol was ;) **Marjan**, ik bewonder je doorzettingsvermogen; het is geen evidentie om na afloop van je contract te blijven doorwerken aan je doctoraat. Hopelijk kan je snel afronden. **Ruth**, ook één van mijn eerste bureaugenoten, later heb ik dan zelfs je bureau geërfd. Het was de beste bureau die ik ooit gehad heb! Wat me aan jou altijd zal bijblijven is je paardjes cursor, haha! En je lieve schattige honden, die niet zo schattige scheten lieten! Het was leuk om je in Bangkok opnieuw tegen het lijf te lopen. **Stefanie**, misschien komen we elkaar nog eens tegen, daar ergens in die stille Kempen. Succes met alles wat doet! **Dorien**, ik heb jou altijd al een sympathieke gevonden. Chapeau dat je je doctoraat nog afgelegd hebt! Ik wens jou en je mooie gezinnetje al het beste toe. **Sofie K**, zotte doos, met jou valt altijd wel iets te beleven! Veel succes nog met de kippenvoeders! **Karolien**, als je goed bent in alles, is het moeilijk kiezen. Ik ben blij dat je je ding gevonden hebt. We hebben samen toch ook heel wat proefjes gedaan, gedeelde smart is halve smart! Bedankt voor alles en veel succes met je praktijk! **Leen T**, jij hebt me voor het eerst getoond hoe een kip te euthanaseren. Het werd toen even zwart voor mijn ogen maar uiteindelijk kwam het goed (voor de kip liep het iets minder goed af). Je stond altijd klaar met raad en daad, bedankt daarvoor! Veel succes met alles wat je doet! **Beatrice**, 't is leuk om je zo hier en daar nog eens tegen het lijf te lopen. Het is altijd een aangenaam weerzien! Ik wens je heel veel succes met het afronden van je doctoraat, het gaat je lukken! **Maxime**/Winnie, mijn favoriete Hollander! (plaats wel te delen met ene Lonneke Onrust) Mijn hart brak een beetje toen je vertrok aan de vakgroep. Het is nog niet zo lang geleden dus de wonden zijn nog vers... Ik moet je bewonderen voor al die zware proeven die je gedaan hebt. Maar het mag niet verbazen want je bent een harde werker! Altijd eerlijk, oprecht en lief. En grappig. Veel succes op je nieuwe werk Maximpie! Maar we zien elkaar nog! Lieve, lieve **Jackeline**, je was het labozonnetje in huis (behalve als je niet goed geslapen had, dan was je een monster). Toen jij wegging, brak mijn hart nog een beetje meer. Ik heb je al heel erg gemist, ook al zit je op nog geen vijf minuten hier vandaan. Ik hoop dat je het naar je zin hebt op je nieuwe werk. Bij deze wil ik me ook verontschuldigen voor mijn cavia mopjes (maar niet echt hahahaha). **Annatachja**, kleine T. rex, 't was fijn om met jou op congres te gaan. Ik zal nooit nog een rollercoaster opstappen zonder aan jouw lijkbleke, verkrampte gezicht te denken. Je bent een super attente bureaugenoot en collega. Heb je ooit al gedacht aan een marktcarrière? Want ik denk dat je aan iedereen je zinksupplementen verkocht zou krijgen. Het is maar een tip ;) Veel succes met je hittestressproef binnenkort! Daarna wacht er je hopelijk nog een reisje naar Tenerife! **Fien**, gij zotte trien! Altijd in voor een mopje of een fratsje. Jij bent één van de meest oprechte mensen die ik ken. Je bent ook één van de meest babbelzieke mensen die ik ken ;) Succes nog met het doorworstelen van je bergen data! Jullie moeten dan gauw eens een spel komen spelen! Kempische **Kirsten**, met het *gerelligste* ochtendhumeur van de hele dienst, je bent een toffe griet met ongelofelijk veel lab skills. Ik hoop dat je nog vele mooie wandelingen mag doen en ontdekken (en dat je die dan doorspeelt ;)). Proficiat nog met de nieuwe woonst, dat je er heel gelukkig mag worden! **Lore**, gij vrouw van't goei leven! Alle restauranttips blijven welgekomen. Ik denk dat je onder andere zelf

een Gault Millau kan uitbrengen. Van mij krijg je alvast een ster of drie voor je persoonlijkheid ;) **Laura**, altijd leuk als oude, toffe bekenden terug op het toneel verschijnen. Heel veel succes met je doctoraatsverdediging. Maar eerst nog een zalige kerstvakantie tijdens de welke je niet aan het werk hoeft te denken! **Marisol**, *pequeña princesa*, je bent enorm grappig! Bedankt voor je luisterend oor en de mind control mints! Ik wens je een goede reis naar Colombia. Maar wel terugkomen hé?! **Jonas**, you crazy Danish guy. It was nice to see you again in Salou. I wish you all the best and lots of success finishing your thesis. **Nathalie G**, nogmaals proficiat met de geboorte van Bastiaan. Veel succes nog met alles wat je doet. **Justine**, jij schattig meisje met de dodelijke uppercut. Ik bewonder je enorme uithoudingsvermogen en doorzettingskracht. Het is jammer dat je wegging aan de vakgroep maar ik ben blij dat je een job gevonden hebt die je volledig ligt. Ik wens je het allerbeste toe! **Pearl**, je eerste proef is ondertussen achter de rug. Hopelijk komt er iets leuk uit! En hopelijk krijg je goed nieuws van het IWT. Je mag nog meer Chinese specialiteiten zoals mooncake meebrengen! **Martina**, you are our newest catch! And we like you! I would like to hear all about your travels! Good luck with your research! **Evelien**, jij maakt er een sport van om de meest uitgebreide *in vivo* proeven te doen. Hopelijk zit het ook eens een keertje mee en wordt je harde werk beloond. Ik ben stiekem jaloers op je kleerkast die vol unieke stukken hangt ;) Succes met al wat je doet! **Lientje**, ik herinner mij nog zeer goed dat ik tijdens één van mijn eerste dagen aan de vakgroep, langs jou kwam te zitten aan de anaerobe kast. Jij aan je kleine Playmobil kastje, ik aan die plastieken Fisher Price. Je leerde me later ook hoe ik gasflessen moest vervangen (waarvoor geen dank). Je bent een sympathieke, vrolijke verschijning die ik al erg gemist heb! **Annemieke**, bedankt om mij in contact te brengen met Steve Baeyen. Het ga je goed daar in Antwerpen! **Steve** & **Annelies**, bedankt voor het uitvoeren van de microbioom analyses maar ook voor jullie tijd en eindeloze geduld om ons alles uit te leggen. Dank ook om mijn manuscript na te lezen en aan te passen. Jullie zijn uiterst sympathiek! **Herman de Rycke**, jou wil ik bedanken voor het lyofiliseren. Op eender welk moment maakte je tijd. Je tip om ampulles te gebruiken om mijn partikeltjes toe te dienen was goud waard! Bedankt ook voor de leuke babbels! **Petra Van Damme** & **Isabel Lemahiu**, bedankt voor jullie hulp bij de osmolariteitmetingen! **Marnik de Norre**, bedankt voor het steriliseren van onze zemelen, je deed dat zonder 'morre' ;) **Kathleen H**, bedankt voor de hulp met de EC aanvragen. **Gunter**, HELD n°1. Ik heb de grootste bewondering voor je dodentochtavontuur. Er zijn er niet veel die kunnen zeggen dat ze hem uitgelopen hebben; maar een paar duizend mensen ongeveer. Per jaar ;) Bedankt voor je hulp met ditjes en datjes! **Jo**, HELD n°2, minstens zo sportief als HELD n°1, bedankt voor je hulp met de papierrompslomp! Maar ook voor de fijne babbels! **Koen**, creatieve duizendpoot, je hebt een talent waar veel mensen jaloers op zijn. Van wat ik al gezien heb, zijn je schilderijen fantastisch mooi. Bedankt om de wegeltjes van Merelbeke te tonen tijdens onze loopmiddagen. Hopelijk vind je je hondje Sabine nog terug. Ik hoor haar geblaf soms nog door de gangen galmen. Lieve **Erna**, de dienst ligt er properder bij dan mijn eigen huis; bedankt dat je zo geduldig bent, in het bijzonder met onze bureau. Ik wens je nog veel ontspannende reisjes naar Oostenrijk toe! Misschien volgende keer met wat meer zon en minder sneeuw ;) **Marc V**/Bompie, voor je leeftijd ben je nog flux en goed te been. Het jong geweld van de vakgroep kan je nog makkelijk af. Ge zijt een toffe peer met een exquis gevoel voor humor :) Bedankt dat ik steeds beroep mag doen op je kippenkennis! Lieve **Marleen**, bedankt voor de organisatie van al die leuke vakgroepuitstappen de afgelopen jaren. Bedankt voor al die pakjes die je ooit verzonden hebt. Ik wens jou en je familie warme kerstdagen toe! **Chana**, merci voor alle hulp tijdens de staalnames maar vooral 10 000 keer merci om mee

kippen te gaan vangen in Nevele; het zijn immers jouw beste vrienden niet :) Het werd enorm geapprecieerd. Probeer in de toekomst ook niet meer van je paard te vallen, we zouden je hier geen tweede keer voor zo'n lange periode kunnen missen ;) **Sarah**, nogmaals proficiat met je verloving. We moeten dringend eens werk maken van ons dubbel matchke hé! Bedankt **Sofie DB** voor je hulp en tips. Als je het antwoord schuldig moet blijven, dan weet je meestal wel waar of bij wie het te gaan zoeken! Sofie, idem als Sarah, we moeten dan dringend eens een plein vastleggen om te gaan tennissen. **Nathalie VR**, bedankt voor alles! Als ik nog eens iets moet flamberen, weet ik je te vinden! ;) **Arlette**, nu officieel de 'oma' van de dienst ;) Nogmaals proficiat met de geboorte van je eerste kleinzoon! Bedankt voor alles! Jij hebt echt een neus voor bacteriën! ;) Handige Harry, ik bedoel, **Serge**, als ik ooit een huis zou verbouwen, dan kom ik bij jou ten rade. Ik denk niet dat er iets is dat jij niet kan. Maar misschien moet je wel stoppen met op je vingers te hameren en van ladders te vallen? ;) Merci voor alles! **Julie**, een echt meisje van 't zeetje! Ik vind je een coole griet! Binnenkort samen een toertje lopen? **Frank**, liefste peter, ik besef dat ik niet altijd een voorbeeldig petekind ben geweest maar ik kan van jou als peter hetzelfde zeggen, ha! Toch heel erg bedankt voor je steun in de laatste rechte lijn van dit doctoraat, it is much appreciated! **An**, jij bent de meest atletische pottenbakker die ik ken, onvermoeibaar! **Filip B**, bedankt om me verder te helpen met antibioticum-gerelateerde problemen, de hulp met de MALDI en de bioveiligheidsopleiding! **Chloë**, je hebt de reismicrobe goed te pakken. Op dit eigenste moment ben je op weg naar Nieuw Zeeland. Ik kan niet wachten om je achterna te reizen! Spreken we af in't midden? **Eva**, stervoetballer, jij kan Krabsla volgens mij in je eentje aan! Bedankt voor de leuke babbels! **Caroline B**/Blaecher/liefste loopvriend, we hebben samen wat afgelopen: de havenloop, dwars door Gent, de midzomernachtrun, 52368 toertjes rond de faculteit… Dan hebben we samen ook heel wat afgewandeld in Rome. Ik heb je al erg gemist sinds je weg bent. Hopelijk hebben we binnenkort allebei terug tijd om iets leuk te doen! Ik kijk er alleszins al naar uit! **Lieze**/Oscaaar, ik wens jou gigaveel succes met je verdediging en de voorbereidingen van je diplomat examen! Je bent een straffe madam, dus dat lukt je allemaal wel! Met twee vingers in de neus! Bedankt om altijd zo enthousiast te zijn, dat werkt aanstekelijk! **Gwij**, ik duim mee voor je post-doc! **Elin**, fellow – braceface, bedankt om me verder te helpen met mijn *Salmonella*-gerelateerde problemen! Je bent een coole! **Connie**, bedankt voor al je tips & tricks. Ik bewonder je en ben stiekem jaloers op die reizen die je al gemaakt hebt! **Delphine**, bedankt voor de hulp bij het snijden en inbedden van de coupes! Maar ook voor de leuke babbels tijdens het versnijden van de weefseltjes. Ik beloof terug meer in de keuken te komen eten nu die doctoraatsperikelen van de baan zijn! **Joachim**, wij ontmoetten elkaar al in 2009 op de Ledeganck en ik was aangenaam verrast om je een aantal jaar later terug tegen het lijf te lopen op de Pathologie. De tand des tijds lijkt geen vat te hebben op je babyface waardoor ik je dan ook onmiddellijk had herkend ;) Merci om de boel op te leuken met je zever, gezever! Ik mag hopen dat je ondertussen ook al de onderste laag van je tuin hebt blootgelegd ;) **Astra**/Astrid, je bent een rare, maar ik hou van raar. En van je sarcastische opmerkingen! Merci voor de hulp bij allerlei praktische dingen en je steun de afgelopen tijd. Ik wens jou en je gezinnetje al het beste toe. **Sarah**, bedankt om me indertijd te helpen met de immuno's! **Christian**, bedankt voor al je hulp met coupes inbedden/snijden/kleuren. Jouw werk lijkt je hobby te zijn, en dat is bewonderenswaardig. Maar vergeet zeker geen tijd te maken voor je andere hobby: reizen. Bedankt ook voor het plantje uit de prehistorie, ik hoop het te propageren tot het einde der tijden ;) **Evelien de Swaef**, leuk om je na onze biologieopleiding weer tegen het lijf te lopen! Doe de groeten aan Diemer en nogmaals proficiat met de geboorte van jullie

zoontje Lars! **Maaike**/Vissenvrouwtje, niemand heeft meer geeky truien dan jij :) Maar ik begrijp het, je bent een vissenvrouwtje of je bent het niet! Je moet ondertussen ook al enorm sterke zeebenen ontwikkeld hebben! Ik wens je heel veel succes met je experimenten en coole transplanten! **Leslie**, je hebt een heel aangename persoonlijkheid! Ik wens jou, Wander en Marilou al het geluk dat jullie verdienen! **Veronique**, ik bewonder hoe je je gezinsleven beredderd en daarnaast dan nog eens zo veel werk verzet! Hoedje af! Bedankt voor de aanmoedigingen in de laatste lijn naar mijn verdediging! **Michiel** & **Leen**, merci om die 27842 bakken aan te brengen tijdens de staalnames en om altijd jullie beste tafel voor ons te reserveren. Het wordt heel erg geapprecieerd!

Ook bedankt om zulke goede collega's te zijn: **An G.**, **Pudsa**, **Alex**, **Keeley**, **Tijn**, **Pudsa**, **Tom**, **Ilse, Jasmien, Helena, Norbert, Han, Laurien, Annelies, Leen VB**, **Prof. Chiers**, **Prof. Decostere**, **Moira**, **Queenie, Tommy, Illias,**... En dan ten slotte, uit het oog maar niet uit het hart: **Nele**, **Iris**, **Johan**, **Myrthe**, **Hannah**, **Lieven**, **David**, **Miet**, **Lien de Cooman**, **Hanne**, **Francesca**, **Sergio**, **Deon**, **Bram**, **Ellen**, **Shaoji**, **Guangzhi**, **Pascale**, **Mark**, **Roel**, **Jordy**, **Cynthia**, **Alexander**, **Magda**, ...

Mij aansluiten bij TC Merelbeke is het beste idee dat ik de afgelopen jaren gehad heb. **Marjolein**, **Kaat**, **Anke**, **Petra**, **Sofie**, **Kathleen**, **Martine**, **Valerie**, **Lies**, **Daan** & **Marco**. Jullie zijn de *Venussen* en *Rogers* van Merelbeke en omstreken! Eén voor één toptalenten en uiterst sympathiek. Ik kan niet wachten tot de interclub in het voorjaar weer van start gaat en kijk al uit naar onze zomerse dubbelmatchkes. Daan, MERCI MERCI MERCI om mijn cover te maken. Hij is echt heel schoon!

Dan zijn daar de bloemschikkers... **Jef**, **Elie**, **Biszy**, **Sven**, **Jochen**, **Ken**, **Ruben** & **Melissa**, **Boone** & **Eva**, dat er nog vele bloemschikweekends mogen volgen, in binnen- en buitenland. Dat we nog vaak mogen gaan eten en feest vieren. **Karolien Persyn**, jij verdient hier een eervolle vermelding. Je bent de coolste griet die ik ken en de beste vriend die ik heb. Elie kon zijn liefke niet beter uitgekozen hebben. Jouw ingefluisterde wijsheid zal mij voor altijd bijblijven: *Ik heb dat nog nooit gedaan, dus ik denk wel dat ik dat kan*. Laten we gauw onze chicks trip plannen!

Alexander/Alejandro/Zaander, proficiat met je nieuwe woonst! Je woont nu net iets verder dan toen we op kot zaten maar dat doet er niet toe. Bedankt om mij kennis te laten maken met de meest bizarre films en om me te vergezellen naar Dranouter. Disco Disco Partizani!! **Kenneth**/Kenny/Frankie, dat je nog door kilo's modder mag fietsen en meters muur mag afklauteren. Proficiat met je nieuwe job! Noor**Jan**/Roostie/Lintworm, ik ben heel erg jaloers op jouw Noorse avonturen & Groenlandse boottochten. Ik wens je veel succes met de laatste loodjes van je doctoraat en hoop je gauw nog eens te komen opzoeken. Misschien moeten we maar eens een tripje naar de Lofoten plannen?

Mama & papa, hoe kan ik jullie ooit bedanken voor alles wat jullie voor mij deden?! Bedankt voor alle kansen die mijn broer en ik kregen. We beschouwden het vaak als vanzelfsprekend maar dat is het niet! Bedankt om de laatste weken zo mee te leven! Ik kijk al uit naar ons reisje naar Lissabon samen! **Moeke & vake**, bedankt dat jullie altijd klaarstonden voor ons, jullie huis is onze tweede thuis. Ik kijk al uit naar het feestje deze zomer! ;) **Koen**, we zien elkaar de laatste tijd te weinig maar ik weet dat je er altijd zal zijn. En dat is wederkerig. Ik ben trots dat jij mijn broer bent. Lieve **Charlotte**, tenniskampioen, onze Koen is met zijn gat in de boter gevallen en ikzelf kan me geen betere schoonzus inbeelden. Tijd om nog eens samen sushi te gaan smikkelen! Succes allebei met jullie doctoraat! **Paul & Nadine**, bedankt om zo mee te leven de

afgelopen weken/maanden en vooral bedankt om zo'n lieve schoonouders te zijn. Ik voel mij echt thuis bij jullie. Dat er nog vele leuke uitstapjes en reisjes mogen volgen! **Jurgen & Elise**, ik kijk al uit naar meer etentjes evenals dansjes in de Charlatan, cocktails in de Gieterij(?) en jenevertjes in 't Dreupelkot. Succes met de nieuwe job, Elise! Je gaat dat goed doen!

En dan ten slotte, **Jonas Loos**, lieve Juun, in veel opzichten zijn we tegenpolen: jij bent doordacht en weloverwogen, ik ben impulsief; jij staat op orde, ik hou van chaos; ik ben vaak rusteloos en jij brengt rust; je bent enorm grappig, ik ben…grappiger ;) Bedankt voor je geduld de laatste weken/maanden(/jaren). Bedankt voor je onvoorwaardelijke steun. Bedankt om al bijna 14 jaar mijn hand vast te houden. Ik zie u graag.
PS, weet dat 'dennis' niet aan je kan tippen, nu niet, nooit! ;)

Karen